The Role of Telomeres in the Development of Cardiac Hypertrophy

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Chapter 1

Literature Review

Overview

Telomeres are non-coding TTAGGG repeats that complex with six key proteins to protect the ends of eukaryotic chromosomes (19, 42) (Figure 1). The number of telomeric repeats, known as the telomere length (TL), varies greatly between species, populations, individuals, and cell types (20, 43). This variation is due to many factors including genetic differences (146), cellular replicative history (60), inflammation (29), oxidative stress (157), and environmental factors, such as excess caloric intake (169) and alcohol consumption (120). Importantly, telomeres must remain above a certain length to prevent the triggering of senescence or apoptosis in all eukaryotic cells (21, 23). As a result of this central role in cell viability, telomere shortening is involved in developmental processes (134), aging (8), and the pathogenesis of age-related diseases (21). Most TL measurements are from circulating leukocytes (120, 155, 169) due, primarily, to the relative ease of acquiring blood samples (9). Emerging evidence, however, of tissue-specific TLs (43, 67, 86), high intra-individual variance in TL (43), and the substantial overlap of risk factors for many diseases and short leukocyte telomeres (52), highlights the need to measure TL in appropriate cell types. This Chapter focuses on TL in the heart and its relevance to heart development, function, and disease (particularly cardiac hypertrophy and heart failure).

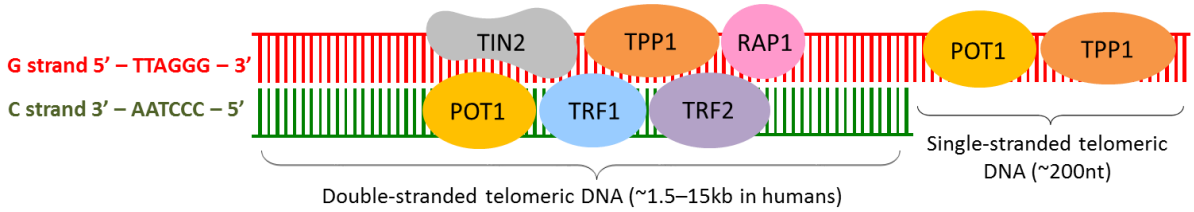

Figure 1. A representation of the telomere sequence and key protective proteins. The 5' vertebrate telomere sequence is TTAGGG and is around 1.5-15 kilo bases (kb) in length in humans and 20-40 kb in most rodents. This includes a single-stranded G-rich overhang of around 200 nucleotides (nt) (19, 20). Six key proteins also bind to the telomere sequence and two to the G-rich overhang to prevent linear ends from being recognised as damaged DNA (42). Image produced using motifolio. TRF1, telomere repeat-binding factor 1; TRF2, telomere repeat-binding factor 2; POT1, protection of telomeres 1; TIN2, TRF1 interacting nuclear factor 2; TPP1, tripeptidyl-peptidase 1; RAP1, repressor/activator protein 1.

Heart disease (also known as cardiovascular disease) is an umbrella term for all heart-related disorders and the worldwide leading cause of death (99). A degenerative and irreversible form of heart disease, known as heart failure, occurs when the heart cannot adequately supply blood to the body (114). This increasingly common disease is caused by the gradual loss of cardiomyocytes (also known as cardiac myocytes, cardiocytes, or simply myocytes in the context of the heart), highly-specialised heart cells that produce the contractions (72, 84, 113, 115). Several other heart diseases, such as atherosclerosis (a hardening and narrowing of the arteries), myocardial infarction (MI) (inadequate blood supply to the heart), and hypertension (high blood pressure) can lead to heart failure through the development of cardiac hypertrophy (discussed below) (50, 155). Furthermore, heart failure can predispose to other heart diseases such as arrhythmias (disorders of impulse generation and conduction) and sudden cardiac death (the abrupt loss of heart function) (47, 155). Therefore, despite this complex interplay, the loss of cardiomyocytes is an overarching pathogenic feature of heart disease and has prompted researchers to identify mechanisms involved in the survival and replication of these cells. As short telomeres lead to senescence and, ultimately, apoptosis (21), several studies have measured TL in cardiomyocytes and heart tissue (Table 1) as a way of providing insights into cardiac function and viability. In this regard, early research showed that TL reflected the replicative history and proliferative potential of rat hearts *in vivo* (71). A following study on human heart failure revealed that cardiomyocytes with telomeres less than 2.5kb in length expressed the senescent marker p16 (also known as $p16^{INK4a}$ and tumour suppressor cyclin-dependent kinase inhibitor 2a) (35). In various strains of mice, the pro-apoptotic transcription factor and tumour suppressor protein, p53, was found to be up-regulated in cardiomyocytes with the shortest telomeres (81, 148). These findings demonstrate that telomere-induced senescence and apoptosis is associated with cardiomyocyte loss and heart disease.

Table 1. A summary of the available articles measuring TL in the heart. Animal studies are listed first and ordered by species, followed by human studies, which are ordered by sample type. The different methods for measuring TL are reviewed elsewhere (102).

Animal models			
Species	**Principle findings**	**Method of telomere measurement**	**Lead author and year of publication**
Fischer 344 rats	Some cardiomyocytes divide throughout life to counteract cell loss. The percentage of cardiomyocytes with short telomeres increases with age.	Confocal microscopy	Kajstura, 2000 (71)
Female Fischer 334 rats	CSC transfusion promotes the regeneration of cardiomyocytes and improves the survival of rats exposed to the anti-cancer drug doxorubicin.	qFISH	De Angelis, 2010 (41)
Male Fischer 334 rats	Age shortens CSC telomeres but some functional CSCs remain in senescent heart and produce young cardiomyocytes.	qFISH and confocal microscopy	Gonzalez, 2008 (57)
Sprague-Dawley rats	Forced *Tert* expression increased cardiac TL and decreased apoptosis.	Southern blot	Oh, 2001 (112)
Sprague-Dawley rats	12-months of low-dose *Staphylococcus aureus* to promote inflammation decreased cardiac TL but did not affect heart function.	qPCR	Raymond, 2014 (126)
Male Sprague-Dawley rats	The antioxidants EGCG, quercetin, and carvedilol prevented cardiomyocyte telomere attrition and apoptosis after abdominal aortic constriction.	Southern blot	Sheng, 2011 (138)
Male Sprague-Dawley rats	Six months of daily beta adrenergic receptor activation decreased left ventricle function but did not change cardiac TL.	qPCR	Raymond, 2013 (125)
Male Sprague-Dawley rats	Ethanol in the drinking water caused cardiomyocyte apoptosis and LV dilation but did not affect cardiac TL.	qPCR	Raymond, 2016 (124)
Male Dahl salt-sensitive rats	Repetitive hyperthermia attenuates cardiac telomere attrition in salt-induced hypertension.	Dot blot	Oyama, 2012 (117)
DahlS.Z-Lepr^{fa}/Lepr^{fa} rats	TL in cardiomyocytes did not differ between lean and obese salt-sensitive rats.	Southern blot	Takahashi, 2014 (144)
Male Otsuka Long-Evans Tokushima fatty rats	Insulin resistant rats present with increased cardiac fibrosis and decreased telomerase activity (which can be ameliorated with PPAR-γ activation) but no changes in cardiac TL.	Dot blot	Makino, 2009 (88)

Male Wistar rats	The anti-cancer drug doxorubicin induces oxidative stress and shortens cardiomyocyte TL.	qFISH	Maejima, 2008 (87)
Male Wistar rats	Severe renal failure shortens cardiac telomeres similarly to myocardial infarction.	qPCR	Wong, 2009 (164)
Hypertrophic heart rats	Cardiac and cardiomyocyte TL was shorter at the onset of hypertrophy but longer at birth and in the presence of established hypertrophy.	qPCR	Marques, 2016 (91)
H9c2 cells	EGCG inhibited telomere shortening in H_2O_2-induced oxidative injury.	Southern blot	Sheng, 2010 (139)
Terc$^{-/-}$ mice	Telomere shortening impaired cell division and increased cardiomyocyte apoptosis and hypertrophy.	Confocal microscopy	Leri, 2003 (81)
IGF-1 mice	IGF-1 mice have longer cardiomyocyte telomeres, less CSC senescence, and increased cardiomyocyte replacement throughout life.	qFISH and confocal microscopy	Torella, 2004 (148)
C57Bl6 mice	The young mouse heart contains cardiomyocytes with different TLs but the proportion of cells with short telomeres increases with age.	Confocal microscopy	Rota, 2007 (131)
C57/Bl6, *eNOS*$^{-/-}$, and *Tert*$^{-/-}$ mice	Cardiomyocyte TL was the same in 3-week and 6-month old sedentary and exercised mice.	Flow FISH	Werner, 2008 (160)
H/M-SOD2$^{-/-}$ mice	The superoxide dismutase and catalase mimetic EUK-8 inhibited changes typical of dilated cardiomyopathy but did not affect cardiac TL.	Dot blot	Makino, 2010 (89)
CAST/Ei mice	Age-associated cardiac telomere shortening was attenuated after 44 weeks of voluntary exercise.	qPCR and Southern blot	Ludlow, 2012 (86)
friend leukemia virus B inbred strain mice	Pim-1 overexpression transiently increases CSCs TL and proliferation.	qFISH and confocal microscopy	Cottege, 2012 (38)
Male C57BL/6, miR-34a$^{-/-}$, and miR-34a$^{-/+}$ mice	The microRNA miR-34a causes cardiomyocyte telomere attrition and apoptosis.	qPCR	Boon, 2013 (25)
Mdx/*Terc*$^{-/-}$ mice	Only mice with a genetic deficiency of *dystrophin* and *Terc* develop heart disease.	qFISH	Mourkioti, 2013 (106)

FVB/N, C57/BL6, *Tert*$^{-/-}$, and *Tert*$^{+/-}$ mice	Overexpression of *Tert* in the heart rescues short telomeres and increases proliferation in mouse cardiomyocytes.	qFISH	Bär, 2014 (12)
Terc$^{-/-}$ and *p21*$^{-/-}$ mice	Cardiomyocyte TL decreases rapidly in the first 15 days of life, pushing cardiomyocytes out of the cell cycle.	qFISH	Aix, 2016 (3)
Male C57BL/6 mice	The senescent heart is characterised by quiescent CSCs with intact telomeres. Stem cell factor can stimulate CSC proliferation.	qFISH and confocal microscopy	Sanada, 2014 (133)
FoxO1$^{+/-}$ mice	Cardiac TL was decreased in FoxO1$^{+/-}$ mice fed *ad libitum* compared to caloric restricted littermates.	Dot blot	Makino, 2015 (90)
H/M-SOD2$^{-/-}$ mice	EGCG prevented cardiac telomere shortening and prolonged survival of H/M-SOD2$^{-/-}$ mice.	Dot blot	Oyama, 2016 (118)
Male Schlager BPH/2J and BPN/3J mice	Telomere attrition in the heart occurs after the development of hypertension.	qPCR	Chiu, 2016 (36)
Bmal1$^{-/-}$ mice	Antioxidant treatment protects cardiac telomeres from oxidation but does not significantly reduce telomere attrition.	qPCR	Hemmeryckx, 2016 (62)
Dogs	TL did not change in cardiomyocytes that were paced to mimic dilated cardiomyopathy after 1, 3, and 4 weeks.	Southern blot and qFISH	Leri, 2001 (80)
Birds (zebra finches)	TL in the heart is correlated with TL in red blood cells.	qPCR	Reichert, 2013 (127)
Birds (siskins)	Malaria infection shortens cardiac TL in parallel with other major organs.	qPCR	Asghar, 2016 (10)
Zebrafish	Telomerase activity and TL increase after cardiac injury but in telomerase-knockouts cardiac cells do not proliferate and repair the heart after injury.	qFISH and confocal microscopy	Bednarek, 2015 (14)
Human studies			
Sample type	**Principle findings**	**Method of telomere measurement**	**Lead author and year of publication**
Fetuses	TL is the same in the kidney, liver, and heart during early embryonic development but then significantly shortens in the heart.	Modified Southern blot	Ulaner, 2001 (151)
Fetal cardiomyocytes	TL is not associated with the senescence of fetal cardiomyocytes *in vitro*.	Southern blot	Ball, 2005 (11)

Biopsies	Telomere shortening and subsequent senescence in CSCs characterises heart disease.	Confocal microscopy	Chimenti, 2003 (35)
Biopsies	Average CSC TL was 7.5kb, more than three times longer than senescent cells. Autologous CSC infusion post MI decreased infarct area and improved heart function.	qFISH and Flow FISH	Bolli, 2011 (24)
Biopsies	CSCs expressing only IGF-1R had longer telomeres and reduced apoptosis.	Flow FISH	D'Amario, 2011 (39)
Biopsies	The growth properties of CSCs in heart failure are similar to those in the normal heart.	Flow FISH	D'Amario, 2011 (40)
Biopsies and cadavers	Myogenic CSCs increased >13 fold in aortic stenosis. Cardiomyocyte hyperplasia contributes to increased cardiac mass.	Southern blot	Urbanek, 2003 (152)
Biopsies and cadavers	Cardiomyocyte apoptosis is associated with short telomeres, defective TRF2, and Chk2 activation. Interference with TRF2 shortens telomeres in nondividing cardiomyocytes.	Southern blot	Oh, 2003 (113)
Biopsies and cadavers	CSC mitotic index increased 14-fold in chronic and 29-fold in acute infarcts. There were more CSCs with short telomeres in chronic and acute infarcts, reducing the number of functional CSCs.	Confocal microscopy	Urbanek, 2005 (153)
Biopsies and cadavers	CSCs can be isolated and expanded *in vitro* with only a 130bp reduction in TL per population doubling.	Confocal microscopy	Bearzi, 2007 (13)
Cadavers	CSCs with short telomeres produce senescent progeny. Females CSCs have longer telomeres and greater replicative potential than males.	qFISH and confocal microscopy	Kajstura, 2010 (70)
Cadavers	TL is not correlated with tissue turnover *in vivo*. The heart typically has the longest telomeres of all major organs.	Southern blot	Takubo, 2002 (146)
Cadavers	Average yearly telomere attrition rate in the myocardium is 20bp. TL decreased an average of 3bp for every one gram increase in cardiac mass.	Southern blot	Terai, 2013 (147)
Cadavers and live donors	Cardiac TL is not significantly correlated with TL in other organs.	qPCR	Dlouha, 2014 (43)

Explanted hearts	Short telomeres in CSCs are biomarkers of heart failure. TL can be used to sort functional CSCs.	Flow FISH	Cesselli, 2011 (31)

Search terms included ‘telomeres in the heart’, ‘cardiac telomeres’, ‘telomere length and cardiac hypertrophy’, and ‘telomere length in heart disease’. Searchers were performed using PubMed in March 2017. TL, telomere length; *Terc*, telomerase reverse transcriptase RNA component; IGF-1 mice, insulin-like growth factor-1-overexpressing mice; *Tert*, telomerase reverse transcriptase catalytic subunit; *eNOS*, endothelial nitric oxide synthase; TRF2, telomere repeat-binding factor 2; qFISH, quantitative fluorescence in situ hybridization; flow FISH, flow fluorescence in situ hybridization; qPCR, quantitative polymerase chain reaction; H/M-SOD2$^{-/-}$ mice, heart/muscle-specific manganese superoxide dismutase-deficient mice; PPAR-γ, peroxisome proliferator-activated receptor-γ; Bmal1$^{-/-}$ mice, brain and muscle ARNT-like protein-1 deficient mice; ROS, reactive oxygen species; p21, cyclin-dependent kinase inhibitor 1; FoxO1, Forkhead transcription factors of O group 1; DahlS.Z-Lepr^{fa}/Lepr^{fa} rats, Dahl salt-sensitive rats crossed with leptin knockouts; H9c2 cells, immortalised rat embryonic cardiomyoblasts; CSCs, cardiac stem cells; IGF-1R insulin-like growth factor-1 receptor.

Telomere length during heart development

Telomeres can be elongated by telomerase, an enzyme comprised of a catalytic reverse transcriptase, *Tert*, and an RNA component, *Terc* (109). Telomerase is particularly active during embryonic development but decreases after birth in most tissues (21), including the heart (26, 112). In addition to being the primary maintenance mechanism of TL, telomerase is involved in many other cellular processes, including growth (141), proliferation (30, 140), and mitochondrial function (2). As such, some studies have focused on telomerase activity in the heart and neglected TL (26, 111, 129, 150). Nonetheless, several findings highlight the importance of TL in the heart as a stand-alone variable that should be directly measured. For instance, heart disease and other age-associated complications in *Tert*$^{-/-}$ and *Terc*$^{-/-}$ mice are due to short telomeres and not telomerase function (142); decreases in TL in heart cells resulting in apoptosis and heart disease can occur without changes in telomerase activity (41); and exercise training can maintain TL in the aging heart independently of telomerase (86). Changes in cardiac TL may also play an important role in normal cardiac development during embryogenesis. In this regard, TL in the human heart (and other major organs) is 15-17kb at 8 weeks gestation but declines significantly by 17 weeks gestation, while in other organs, such as the kidney and liver, telomeres remain long (151). When grown *in vitro*, however, human fetal cardiomyocytes stop dividing independently of TL after 20-25 population doublings (11). Furthermore, lifespan of cultured human fetal cardiomyocytes remains unchanged even when telomeres are elongated via transduction of *TERT* (11). In contrast, *TERT* overexpression can immortalise many other primary human cells (79) and prolong the survival of adult rat cardiomyocytes (112) by increasing TL. In addition, *Tert*-overexpression specific to the myocardium in mice suppressed telomere attrition and prevented terminal differentiation of cardiomyocytes in the first month of life (112). Therefore, TL may play

important roles in early cardiac development but appears to be independent of the mechanisms controlling prenatal senescence and developmental apoptosis in the heart.

In the first 15 days of life, mouse cardiomyocytes are pushed out of the cell cycle by a rapid decrease in TL, which activates the growth arrest protein p21 (also known as cyclin-dependent kinase inhibitor 1 and $p21^{Cip1}$). Neonatal cardiomyocyte senescence was prevented in p21 knockout mice indicating that TL in the heart may be directly involved in cardiac development after birth (3). These findings offer an explanation for the cardiomyocyte division that has been observed in the first (123) and third (110) weeks of life. Despite significant telomere attrition during prenatal and neonatal development, early findings on human cadavers revealed the heart typically has the longest TL of all the major organs (146). Also in contrast with results from fetal hearts (151), telomere attrition in the left ventricle is relatively slow after the neonatal period, losing only 13bp per year compared to other tissues and organs, such as the kidney, which decrease 30-60bp per year (146). A follow up study by these authors reported a similar telomere attrition of 20bp per year in the myocardium using 530 autopsied human samples and regression analysis (147). In a more recent study on twelve cadavers aged 0-88 years, cardiac TL was non-significantly longer than in the liver, brain, triceps, and skin but was not correlated with TL in any of the eleven organs studied (43). Therefore, tissue-specific patterns of telomere attrition lead to intra-individual variations in TL that differ before and after birth.

Telomere length in cardiac hypertrophy

In neonatal humans only around 0.016% of cardiomyocytes are in cytokinesis and this decreases to undetectable levels in the early twenties (101). Such limited proliferation restricts most cardiac growth after birth to cardiomyocyte hypertrophy (50). Overt cardiomyocyte growth, however, causes an increase in the contractile mass of the heart, known as cardiac hypertrophy (or left ventricular hypertrophy) (45), and is the biggest risk factor for heart failure and sudden cardiovascular death with the exception of old age (Figure 2) (74). Cardiac hypertrophy is also directly linked to cardiomyocyte loss in the diseased heart where the remaining cells increase in size to compensate for the death of their neighbours (115). Interestingly, when cardiomyocytes from 12-week old mice were separated into three categories based on size (< 20 000, 20 000 – 40 000, and > 40 000μm^3) TL significantly decreased (from 40±0.73 to 33±0.82 and then 25±0.36 kb [mean±SEM], respectively) with each increase in cell size (131). These findings demonstrate that cardiomyocyte TL is related to cell size (a necessary feature of development and a key predictor of heart disease) and that TL is not homogenous even within a specific cell type. In accordance with these findings on TL, smaller cardiomyocytes had no detectable senescent markers, while some of the larger, and all of the largest cells, had high levels of p16 expression (131). Although these three categories of cardiomyocytes are present throughout life, the number of senescent and hypertrophied cells increases and the number of young and small cells decreases with age in mice (131) and humans (35). Indeed, telomere attrition in young and old cardiomyocytes leads to the same senescence program but the number of cells involved is significantly greater as age increases (148). Finally, contractile ability (130, 148) and intracellular calcium concentrations (131) also decrease in the presence of short telomeres and hypertrophy. Taken together, these findings suggest that cardiac aging is

characterised by short telomeres, hypertrophy, senescence, and decreased contractile performance all at the cardiomyocyte level.

The inverse association between TL and both cardiomyocyte development and cardiac hypertrophy suggests that the loss of telomeric repeats may affect cardiomyocyte function before senescence or apoptosis is reached. Indeed, cardiac telomere attrition *in utero* (151) coincides with the heart becoming functional (5) in humans. Moreover, the rapid telomere attrition that occurs when cardiomyocytes withdraw from the cell cycle (3) heralds the switch from hyperplastic to hypertrophic growth (110, 123) in neonatal mice. In further support of this suggestion in humans, individuals of African descent have longer telomeres at birth (59), an increased rate of (leukocyte) telomere attrition in adulthood (66) and are more prone to heart disease than Caucasians (167). In addition, men have shorter telomeres in the heart (70) and other organs (55) as well as a greater incidence of heart disease than women (165). Furthermore, twin studies have estimated that 84% of changes in TL (in leukocytes) are attributable to genetic factors (69). Similarly, twin and family studies have estimated the heritability of cardiac mass to be 30–84% (28, 94, 137), with primary cardiac hypertrophy affecting up to 16% of European- and 43% of African-descendants (54). Collectively, this suggest that TL, rate of telomere attrition, cardiac hypertrophy, and heart disease are significantly determined by genetic factors.

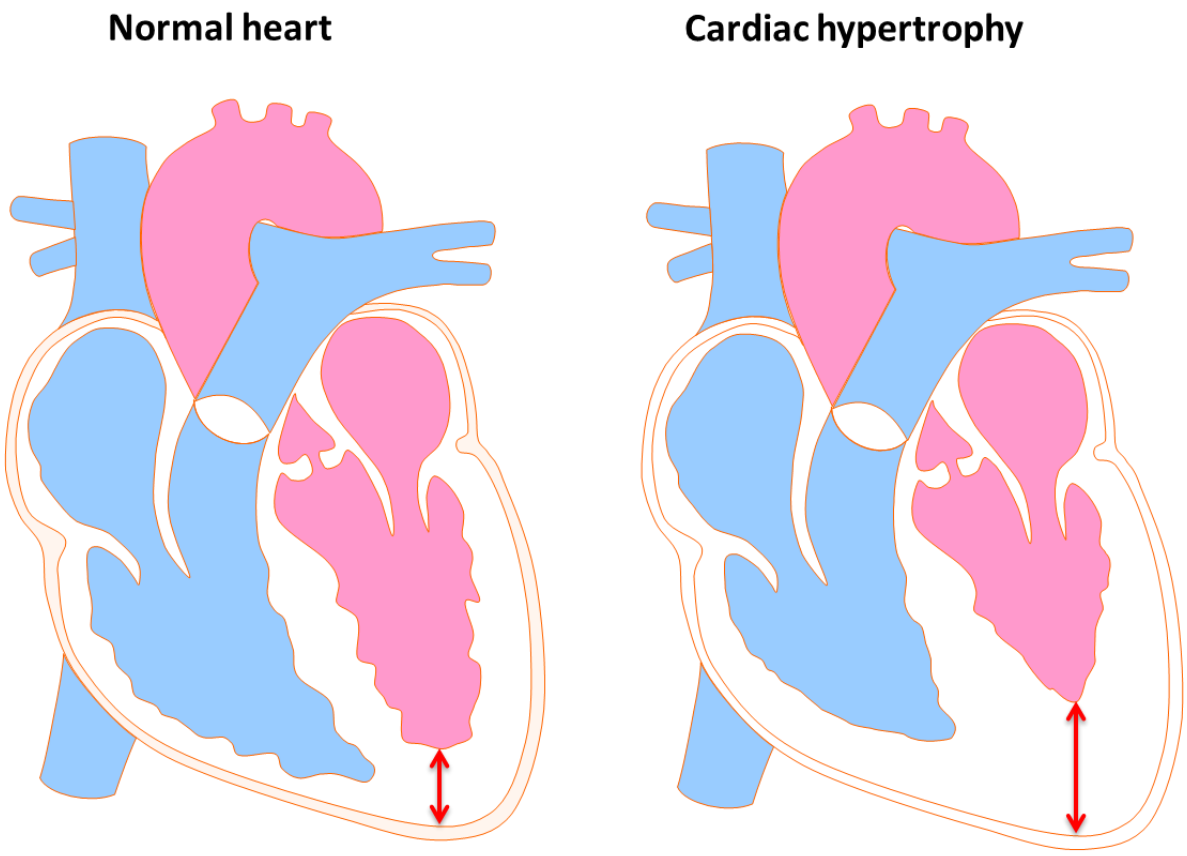

Figure 2. The normal heart compared to the hypertrophic heart. Cardiac hypertrophy involves a thickening of the ventricular wall (compare red arrows) which causes a maladaptive decrease in chamber size (compare lower pink areas) and, consequently, cardiac output. As cardiac hypertrophy primarily affects the left ventricle, it is also known as left ventricular hypertrophy.

Cell-specific telomere length in the heart

In addition to shorter telomeres, cardiac hypertrophy, senescence, and apoptosis, fibrosis is a hallmark of aged hearts in rodents (25, 88) and humans (44). Fibrosis occurs when cardiac fibroblasts, which synthesise the extracellular matrix to support cardiomyocyte contraction (158), deposit excess collagen in place of cardiomyocytes that have undergone cell death (35). Cardiac fibroblasts were originally believed to constitute 60-70% of all cardiac cells and 30-40% of the heart's mass, with cardiomyocytes accounting for 70-80% of cardiac mass but only 30-40% of all heart cells (108). While estimates of the cardiomyocyte population have not changed (18), a recent report using cell-specific markers found that endothelial cells were the most numerous cell type in the heart and account for around 45% of all cardiac cells (122). Relatively small numbers of leukocytes, cardiac stem cells (CSCs), vascular smooth muscle cells, monocytes and macrophages have also been detected (40, 108, 122). Interestingly, TL in the heart has only been measured in isolated cardiomyocytes, CSCs (discussed below), and whole heart tissue; separated endothelial cells or fibroblasts, as far as the literature revealed, have not been studied. The consensus that heart disease is a result of cardiomyocyte insufficiency, has led some authors to argue that TL in whole heart tissue from non-infarcted areas (which contain less collagen than infarcted areas (76, 143)) reflects that of cardiomyocytes (164). This however remains to be shown and as TL significantly varies in cardiomyocytes from the same individual (131), cell specific TL measurements should be considered the most valid, followed by sorted cells, whole tissue, and lastly the use of circulating leukocytes as a proxy (the available research that measured TL in heart cells, along with the specific cells used, is summarised in Table 1). Nonetheless, the measurement of telomeres in cardiac fibroblasts and endothelial cells under different conditions could help elucidate the complex intra-individual variation in TL and how it affects the heart.

Telomere length in heart failure and function

As with heart development, functional changes associated with cardiac injury are also accompanied by changes in telomeric variables. For instance, protein levels of *TERT*, the rate limiting step of telomerase (83), increased 8.6 fold during acute and 2.6 fold after chronic MI in humans (153). Similarly, in male Sprague-Dawley rats subjected to abdominal aortic constriction, there was an increase in *Tert*-mRNA positive cardiomyocytes during the development of pressure overload-induced cardiac hypertrophy but a decrease in the later stages of heart disease (138). Moreover, in dogs with dilated cardiomyopathy induced by pacing their hearts to 210 beats per minute, Tert protein levels increased in the first three weeks before significantly decreasing after four weeks (80). Interestingly, in each of these cases, cardiac TL either decreased or stayed the same, despite increases in *Tert*. This suggests that the heart responds to stress by upregulating *Tert* to maintain TL. An increase in *Tert*, but unchanged or decreased TL, could also be explained by the enhanced cardiomyocyte turnover that is often present in the diseased heart (80). For instance, two in every thousand cardiomyocytes in human cardiac hypertrophy induced by aortic stenosis were in mitosis and, like Tert levels, cardiomyocyte division was inversely correlated with the duration of the disease once it was present for more than one year (152). Moreover, total Akt is similar in both healthy and infarcted human hearts but active phosphorylated Akt increased 21-fold during acute myocardial infarction and 3.8-fold after chronic myocardial infarction. Finally, Akt also activates *Tert* (46) resulting in a transient increase in telomerase and proliferation at the onset of heart disease. Taken together, these findings demonstrate that acute stress on the heart induces compensatory mechanisms aimed at preserving TL. As the heart disease progresses, however, these mechanisms become attenuated and then exhausted leading to overt cardiac failure (35, 113).

In end-stage human heart failure, average cardiomyocyte TL is 25% shorter than age-matched controls (7.8+-0.2 and 6.5+-0.2kb respectively) (113). In contrast with findings on mice (25, 131) and rats (91), significant telomere erosion only occurred in the presence of heart failure and not cardiac hypertrophy alone (113). Similar results were found by Terai and colleagues (147) with each one gram increase in heart weight corresponding to only a 3bp decrease in TL. Moreover, individuals who died of heart disease had the heaviest hearts and the shortest telomeres but this was not always significant (147). These modest changes, however, could be explained by the measurement of TL in whole heart tissue compared to isolated cardiomyocytes in the other studies discussed so far. In this regard, one study found that average cardiomyocyte TL was 39% shorter in diseased compared to normal aged human hearts (35). Furthermore, cardiomyocyte apoptosis increases 0.5-1% in end-stage human heart failure (72, 113). While such low percentages may seem insignificant, Wencker and colleagues (159) found that a rate of apoptosis of 0.023% was pathologically relevant and these findings could be explained by decreases in cardiac TL. Therefore, there may not be a net decrease in TL at the tissue level even if there is sufficient telomere-induced cell loss to cause heart failure and cell-specific TL measurements may be necessary to determine organ viability. Uncovering the role of telomeres in normal cardiac growth and in the transition to cardiac hypertrophy and failure could help to identify harmful factors that could be manipulated to treat heart disease.

Factors that exacerbate cardiac telomere attrition and heart disease

Defective telomerase

In the two most common models of telomere dysfunction, *Tert* and *Terc* knockout mice, a lack of telomerase activity causes cardiomyocyte TL to shorten 3-5kb per generation, with cardiac hypertrophy and heart failure presenting by the second and fifth generations respectively (22, 81). As early generations of these mice are healthy, this strongly suggests that short TL (which can also be viewed as ineffective telomere maintenance) and not defective *Tert*, *Terc* or telomerase expression, is the cause of the cardiac pathologies that occur in these animals. These (inter-generational) changes also parallel the stages of human heart disease and *Terc* knockout mice are considered a good model of human heart failure (163). Systemic telomere inhibition, however, creates several comorbidities that affect the heart making it impossible to determine the role of TL in heart function. For instance, telomere shortening in endothelial cells can cause atherosclerosis (100) and third generation *Terc* knockout mice have high blood pressure (121) before the onset of established cardiac dysfunction (20, 81). Importantly, these conditions can affect cardiac telomere attrition through pressure induced hypertrophy and heart failure (50, 161). Interestingly, haploinsufficency of both *Tert* and *Terc* in mice causes telomere erosion and limits tissue renewal to the same extent as *Tert* and *Terc* knockouts (142) (although other research suggests this is only true for *Terc* knockout mice (34)) indicating that the only critical function of telomerase is telomere elongation. Similarly, in humans, single mutant *TERT* and *TERC* alleles can cause heterogenous phenotypes within families (119). Moreover, these individuals are asymptomatic in early life, indicating once more that short TL, and not defective telomerase expression, determines disease.

Insufficient telomeric proteins

There are six key proteins, collectively named shelterin, that directly bind to the telomeric DNA sequence to constitute a telomere (42) (Figure 1). As shelterin is needed to protect telomeres, a paucity of these proteins can cause rapid telomere erosion. For instance, *Tert* and *Terc* knockout mice are viable until the fifth generation (20), however many knockout mice deficient in a shelterin gene, such as telomere-repeat binding factor 2 (*TRF2*), are embryonically lethal (92). Indeed, a decrease in *TRF2* expression has been suggested as the earliest event in some forms of telomere shortening (107). In human heart failure, telomere attrition is associated with decreased expression of *TRF2* and an increase of the pro-apoptotic mediator Checkpoint kinase 2 (*Chk2*) (113). In cultured noncycling mouse cardiomyocytes, suppression or antisense knockdown of *Trf2* caused telomere erosion and apoptosis (113). Conversely, because shelterin proteins are subunits of telomeres (42), short TL may also reduce *Trf2* levels. In support of this notion, decreased TL and *Trf2* expression (and Chk2 activation) is prevented in cardiac-specific *Tert* transgenic mice (113). Transcriptome changes that influence cardiac TL and Trf2 expression have also been traced back to microRNAs, noncoding RNA molecules that post-transcriptionally regulate gene expression (105). For instance, MicroRNA-34a$^{-/-}$ mice showed less cardiac aging, hypertrophy, and cell death compared to wild type controls (25). One target of microRNA-34a is protein phosphatase 1 regulatory subunit 10 (also known, and hereafter referred to, as *Pnuts*), which facilitates the binding of *Trf2* to telomeric DNA (75). In line with the post-transcriptional action of microRNAs, *Pnuts* expression in microRNA-34a$^{-/-}$ mice was more down-regulated at the protein level (25). Furthermore, *Pnuts* overexpression in cultured mouse cardiomyocytes reduced apoptosis and telomere attrition without changing telomerase activity (25). This highlights the importance of telomeric regulators and their utility in preserving cardiac TL and function.

Biomechanical stress

The most common cause of cardiac hypertrophy and heart failure is other heart diseases, particularly hypertension (50) and atherosclerosis (161), which place biomechanical stress on cardiomyocytes (74). In male BPH/2J mice, a genetic model of hypertension, cardiac TL was not different from normotensive controls at 4 weeks of age before the onset of hypertension but became significantly shorter at 12 and 20 weeks of age after the development of high blood pressure (36). In male Sprague-Dawley rats subjected to abdominal aortic constriction (to mimic atherosclerosis), heart weight, telomere attrition, p53 levels, and cardiomyocyte apoptosis increased progressively (138). In both these cases, which model the two most common causes of human heart failure, it appears that cardiac telomere shortening is a by-product and not a cause of the disease. It is still possible, however, that telomeric factors, such as Trf2 insufficiency, are involved in the onset of the disease, before changes in TL are observed. For instance, it has been demonstrated that telomere uncapping in leukocytes influences hypertension status whereas mean TL does not (103). Uncapped telomeres present with DNA damage known as Telomere dysfunction-Induced Foci (TIFs) (145), which are identified by the colocalization of shelterin and DNA damage response proteins, such as Trf2 and gamma-H2AX (98). Indeed, although telomeres must maintain a critical length to allow the formation of protective loops (Figure 3), telomere structure is the most important factor governing cell viability (19). In support of this, it has also been suggested that apoptosis caused by Trf2 insufficiency may be independent of TL (92). Perhaps studies of telomere structure, or the identification of novel telomeric proteins, could elucidate the link between heart function and TL.

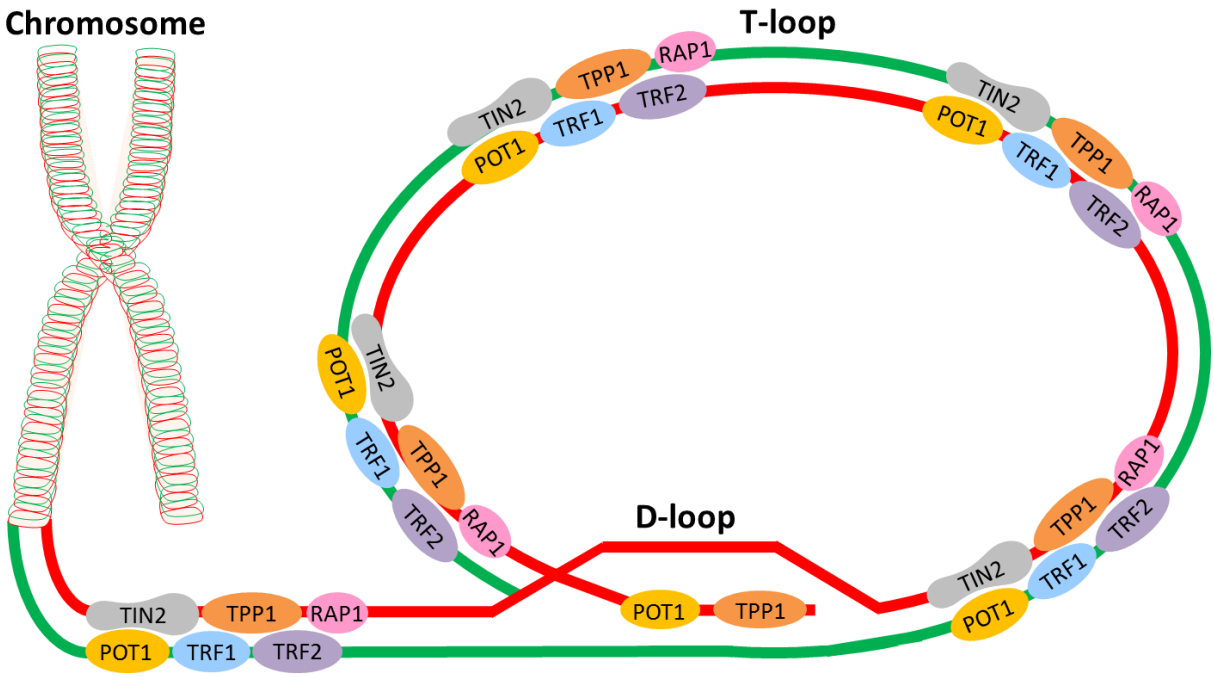

Figure 3. A representation of the telomere structure. The formation of protective T and D loops prevents cellular machinery from recognising telomere ends as damaged DNA (42) (only one of four telomeres on the chromosome is shown). Image produced using motifolio. TRF1, telomere repeat-binding factor 1; TRF2, telomere repeat-binding factor 2; POT1, protection of telomeres 1; TIN2, TRF1 interacting nuclear factor 2; TPP1, tripeptidyl-peptidase 1; RAP1, repressor/activator protein 1.

Renal Failure

Renal failure is another common disease that often leads to adverse cardiac remodelling (4). Indeed, severe renal failure in rats shortened TL in the heart by around 20% and induced cardiac hypertrophy (164). A key pathophysiological feature of heart failure (149) is beta adrenergic receptor activation, which can be traced back to the well-studied Renin Angiotensin Aldosterone System (RAAS) (reviewed elsewhere (116)). Beta adrenergic receptor activation also induces cardiomyocyte hypertrophy (97) and adrenergic receptor activation with Angiotensin II (AngII) has been shown to decrease TL in the heart (39). In male Sprague Dawley rats, beta adrenergic receptor activation with isoproterenol for six months increased left ventricle dysfunction but did not affect cardiac TL or hypertrophy compared to saline-treated controls (125). Consequently, the authors concluded that beta adrenergic receptor activation is likely not responsible for the telomere attrition observed in heart failure. Although blood pressure was not measured in this study, Sheng and colleagues (138) also used male Sprague Dawley rats and found that the beta receptor blocker and anti-hypertensive drug carvedilol reduced cardiac hypertrophy, telomere attrition, and Trf2 protein loss in these animals. Other research on rats, however, has also failed to associate cardiac dysfunction with hypertensive status (91, 124). Therefore, RAAS activation and interactions between the heart and kidney can influence both cardiac TL and function but this may occur independently of hypertension.

Hypoxia

MI also leads to heart failure, telomere attrition and cardiac hypertrophy. In rats, MI caused a similar reduction in TL as renal failure (around 20%), however, renal failure and MI in combination did not exacerbate telomere shortening (164). Hypoxia-induced cardiomyocyte necrosis is primary consequence of MI (32) and severe hypoxia (95% N_2 and 5% CO_2)

reduces cell number and alters morphology in cultured rat cardiomyocytes (68). Interestingly, necrosis is even more significantly associated with age than apoptosis in cardiomyocytes from wild-type (WT) and transgenic mice (148). As a corollary, necrosis is correlated with oxidative stress, hypertrophy, age, and p16 and p53 expression in normal mice and those with longer lifespans (148). Although necrosis is not considered telomere-dependent, this suggests that necrotic debris may affect TL and survivability of proximal cardiomyocytes and could be a mechanism of cell loss with age and particularly after MI. In this regard, MI can destroy up to 25% of the adult heart (77), however only 1% (17) to 1.9% (101) of cardiomyocytes are replaced annually in a 20 year old person, and less than half of cardiomyocytes present at birth are replaced in an average lifetime (17). This innate limitation highlights the importance of identifying the primary causes of cardiomyocyte loss to prevent and treat the many types of heart disease.

Oxidative stress

The slow turnover of cardiomyocytes means that telomere attrition must be due to mechanisms other than cell division. The most common explanation for this phenomenon is oxidative stress (2, 51, 81, 156) and several findings support this suggestion. For instance, oxidative stress and telomere attrition increase in the presence of heart disease in Sprague-Dawley rats (138) and WT mice (148). In addition, more that 80% of apoptotic human cardiomyocytes exhibit oxidative damage (153). Oxidative stress also plays a particularly important role in the development and outcomes of diabetes and metabolic syndrome (obesity, insulin resistance, and hypertension) in humans (51, 156). Animal models of diabetes and metabolic syndrome, however, do not always exhibit increased cardiac telomere attrition. For instance, in Dahl salt-sensitive (DS) rats crossed with Zucker rats with a missense mutation for the leptin receptor gene (obese) (DS/obese rats) to mimic metabolic syndrome, oxidative stress accompanied cardiac hypertrophy and dysfunction at 18 weeks of

age (144). TL, however, did not differ between the DS/obese rats and lean homozygous controls despite the upregulation of Chk2, p21, and p53 (144). Similarly, although Tert and Trf2 protein levels were decreased, no changes in cardiac TL were found at 40 weeks of age in a rat model of type 2 diabetes (88). Nonetheless, these findings suggest that oxidative stress can cause telomere erosion in the heart by directly interacting with telomeres as well as decreasing telomerase activity and Trf2 protein levels.

Inflammation

Another potential factor to explain cardiac telomere attrition in the absence of significant cell division is inflammation (82, 93, 126, 156). In this regard, Sprague-Dawley rats exposed to a low-dose of *Staphylococcus aureus* for 12-months to promote inflammation had decreased cardiac TL but no change in heart function (126). Determining the effects of oxidative stress and inflammation in heart disease, however, is challenging as they often occur in conjunction (117, 144) and can induce one another (65). It has also been proposed that oxidative stress and inflammation are a result, and not a cause, of heart disease (168) although studies that have induced oxidative stress (62) and inflammation (126) suggest a causal involvement. Nevertheless, mitochondrial compromise can exacerbate oxidative stress (58) and decreases in (leukocyte) TL (132). As such, it has been suggested telomere attrition and cardiac dysfunction is a consequence of decreased cardiomyocyte mitochondrial biogenesis (104). While it is possible that a third confounding factor, such as mitochondrial compromise, could cause decreased cardiac function and TL, the most probable explanation is that several pathways interact with telomeres and collectively contribute to cellular dysfunction in the heart. Their relative importance and the causal agents likely depend on the specific type of disease as well as the genetic and environmental factors involved.

Environmental toxins and unhealthy lifestyle

Several environmental toxins, including tobacco and alcohol, along with lifestyle factors, such as sedentary behaviours and mental stress, are also associated with heart disease and telomere erosion (52). Of these, however, only the effects of alcohol consumption on cardiac TL have been assessed. Ethanol administration (5% v/v in drinking water) to rats for four months increased cardiomyocyte apoptosis and LV dilation but there was no change in cardiac TL, blood pressure, heart weight, C reactive protein (a marker of inflammation), or fasting blood glucose (a marker of diabetes) (124). Although these results suggest that TL is not involved in the cardiac dysfunction observed in this model, it is also likely that four months may not be sufficient to cause a significant change in cardiac TL. Nonetheless, these diseases highlight the sensitivity of the heart to a myriad of stimuli and comorbidities that can damage cardiomyocytes and lead to heart disease. TL, however, has also been used as a proxy for systemic stress. For instance, malaria infection has been shown to decrease cardiac TL in parallel with other major organs in birds (10). Although intra-individual variations in TL are less pronounced in birds (127) than humans (43), these authors considered short TL in multiple organs (including the heart) a marker of systemic stress (10) and thus not necessarily of heart disease in particular. The primary stressor of cardiomyocytes is considered to be biomechanical stress (50) and aortic constriction to induce this significantly reduces TL in mice and humans (113). Collectively, this highlights the need to determine and control for 'stressors' that exacerbate heart disease and telomere attrition so the exact involvement of telomeres in the heart can be uncovered.

Factors that protect cardiac telomeres and heart function

Antioxidants

Antioxidants can prevent the damaging effects of oxidative stress on telomeres and cardiomyocytes. For instance, Bmal1$^{-/-}$ mice (a model of premature aging due to increased ROS production) exposed to a high fat and high cholesterol diet, suffer from increased cardiac telomere attrition and oxidation (62). Ten weeks of treatment with the antioxidant TEMPOL, however, significantly decreased telomere oxidation and non-significantly attenuated telomere attrition (62). Similarly, Makino and colleagues (89) examined telomere biology in heart/muscle-specific manganese superoxide dismutase-deficient mice (H/M-SOD2$^{-/-}$), which develop premature heart failure. EUK-8, a superoxide dismutase and catalase mimetic with antioxidant properties, was administered to H/M-SOD2$^{-/-}$ mice for four weeks beginning at 8 weeks of age (89). At the end of the study, no shortening of TL was observed in heart tissues from all mice tested, but telomerase activity and Trf2 protein levels were downregulated in H/M-SOD2$^{-/-}$ mice and this could be inhibited by EUK-8 treatment (89). The short duration of this study may explain why there were no changes in TL. Indeed, the administration of the powerful antioxidant epigallocatechin gallate (EGCG) to H/M-SOD2$^{-/-}$ mice for 8 weeks (as opposed to only 4 weeks (89)) prevented the dilated cardiomyopathy, increased inflammation, and decreased TL observed in the control group (118). EGCG and another antioxidant, quercetin, also inhibited oxidative stress, TRF2 protein decreases, and telomere attrition in male Sprague-Dawley rats subjected to abdominal aortic constriction (138). Similar results have also been found in cultured mouse cells with exogenous Trf2 (113) and Pnuts-overexpression (25) conferring protection form oxidative stress. Moreover, EGCG inhibited decreases in TL and protein expression of Trf2 in immortalised rat embryonic cardiomyoblasts (H9c2) cell line induced by oxidative stress

(139). Finally, activation of the mitochondrial regulator peroxisome proliferator-activated receptor-γ (PPAR-γ) was found to ameliorate cardiac oxidative stress, as well as *Tert* and *Trf2* decreases without changes in TL at 40 weeks of age in diabetic rats (88). These 'rescue' studies provide further evidence that decreasing oxidative stress protects cardiac telomeres and function.

Caloric intake

Excess caloric intake is the cause of obesity and the primary risk factor for diabetes (7), both of which can lead to heart disease and (leukocyte) telomere attrition (73, 169). Early research showed that calorie restriction (CR) decreased the mortality rates of rats at 800 days of age from 50 to 80% (16). In this regard, cardiac TL was significantly shorter in heterozygous Forkhead transcription factors of O group 1 ($Fox01^{+/}$) (homozygous knockouts are embryonically lethal) compared to WT mice at 35 weeks of age when fed *ad libitum.* However, CR (70% of *ad libitum)* for 20 weeks from 15 weeks of age prevents the accelerated TL in $Fox01^{+/-}$ mice (90). CR also reduced oxidative stress and cardiomyocyte apoptosis in WT mice (90). It should be noted, however, that heart to body weight ratio or blood glucose levels in CR and *ad libitum* WT and $Fox01^{+/-}$ mice did not change, limiting the applications of these findings to human obesity and diabetes (and the subsequent cardiac complications). Importantly, the most powerful preventative strategy for obesity, diabetes, and heart disease is simply a healthy diet (such as sufficient antioxidants and reduction of fat) combined with adequate exercise (162). In this regard, however, cardiomyocyte TL in mice did not change after 3 weeks or 6 months of exercise training. Interestingly, control animals over 18 months of age showed significant telomere shortening (160) suggesting that, as in models of heart disease (62, 89), exercise-induced effects on TL also take time to become significant. In support of this notion, age-associated cardiac telomere shortening was attenuated after a more prolonged 44 weeks of voluntary exercise in rats (86).

Exercise

Despite a delay in changes to cardiac TL, many rapid responses to exercise involve telomeric pathways. For instance, Tert and Trf2 protein levels increase and p16, p53, and chk2 protein levels decrease in mice after three weeks of voluntary running (160). Interestingly, these exercise-induced changes were absent in *Tert*$^{-/-}$ mice (160). Similarly, WT mice supplied with running wheels are also immune from the cardiotoxic effects of the anti-cancer drug doxorubicin whereas *Tert*$^{-/-}$ mice are not (160). In addition, cardiac-specific overexpression of *Tert* promotes survival after myocardial infarction and protects against cardiomyocyte telomere attrition and apoptosis (112). Akt (130) and IGF-1 (148) overexpression in mice also causes an increase in the number of small cardiomyocytes with greater contractile performance and longer telomeres leading to increased LV function and delayed aging. Furthermore, intraperitoneal treatment with IGF-1 in mice increased telomerase activity and phosphorylated Akt protein levels (160), suggesting these pathways may be linked. Moreover, cardiomyocytes in stem cell factor (SCF)-treated mice were smaller with longer telomeres compared to saline-treated controls (133). Interestingly, SCF (48) as well as IGF-1 and Akt (160) are upregulated following exercise and this could be one pathway that promotes exercise-induced cardioprotective adaptations.

Physiological cardiac hypertrophy

Although cardiac hypertrophy is a powerful predictor of heart failure, an increase in cardiac mass can also be an adaptive response to exercise (49) or pregnancy (1). This so-called *physiological* cardiac hypertrophy or athlete's heart (reviewed elsewhere (45, 95)) is functionally and mechanically distinct from the pathological kind in that it is adaptive, reversible, and harmless. Interestingly, pathological hypertrophy is also reversible in its early stages (44) and this coincides with the upregulation of telomeric variables and unchanged TL

seen in the early stages of heart disease (80, 138, 153). In addition, TL is maintained (160) or even extended (86) with regular exercise, suggesting that telomere attrition may be a specific feature of pathological hypertrophy and thus a better marker of heart function than cardiac mass. Furthermore, *Tert* and IGF-1 overexpression in rodents is cardioprotective but also induces hypertrophy in cardiomyocytes (37, 64, 112). As only small cardiomyocytes are capable of undergoing replication (35, 81, 152), physiological cardiac hypertrophy may seem counter-intuitive but IGF-1 and Akt overexpressing mice also possess an increased number of small replicating cardiomyocytes (130, 148). Furthermore, Tert (112), as well as IGF-1 and Akt (128), is involved in cardiomyocyte proliferation, suggesting that adaptive cardiac growth includes hypertrophy and a relative increase in cell number (hyperplasia). Indeed, levels of cardiomyocyte death and replication are similar during aging in IGF-1 mice (148) but cell loss exceeds replication in the hearts of aged WT mice and rats (57). Therefore, it is tempting to speculate that pathways involved in physiological or 'pre-pathological' cardiac growth promote telomere elongation to protect against stress-induced (be it physiological or pathological) cardiomyocyte loss.

Hyperthermia and combining treatments

One final treatment shown to influence TL in the heart is repetitive hyperthermia. In this regard, Oyama and colleagues (117) found that 4 weeks of daily immersion for 10 min in 40°C water prevented cardiac telomere attrition, oxidative stress, and hypertrophy in 10-week old salt-induced hypertensive rats. Interestingly, significant changes in cardiac TL occurred relatively quickly in this study whereas most research suggests that factors regulating TL, such as Tert and Trf2, are likely more relevant in medium- and short-term treatments. Nonetheless, when taken together, these findings highlight the ability of endogenous and exogenous factors to increase cardiomyocyte survival through telomeric pathways. Identifying the molecular mechanisms responsible for the adaptive cardiac changes following

antioxidant treatment, CR, exercise, and hyperthermia could help develop novel treatments for heart disease. In this regard, the effects of regular mild hyperthermia may be similar to those of regular exercise. Furthermore, improved anti-oxidant capacity is also an adaptive response to exercise (78) and physical activity reduces fibrosis in injured rat hearts (82). Given the beneficial effects of exercise on TL and several measures of heart function, identifying the precise mechanisms distinguishing pathological from physiological growth provide promise of shifting pathological cardiac hypertrophy to the physiological end of the spectrum. This could not only rescue TL but also degenerative cardiomyocyte loss. Despite the importance of diet and exercise in preventing and treating heart disease, however, little research has determined the effects of these variables on cardiac TL.

Telomere length in cardiac stem cells

The mammalian heart contains cardiac stem cells (CSCs) that divide throughout the lifespan to replenish old and damaged cardiomyocytes (6). Since this discovery, much research has focused on understanding replication and senescence in these cells to potentiate cardiac repair with age and after injury. *Ex vivo* endomyocardial biopsies obtained from the apex of the left ventricle in humans have revealed two types of CSCs; myogenic CSCs, which constitute 97% of CSCs and mainly form cardiomyocytes, and vascular CSCs, which constitute only 3% of CSCs and mainly form smooth muscle and endothelial cells (40). Like cardiomyocytes, TL regulates the life cycle of CSCs (13) with longer telomeres designating CSCs that are replicating and replacing old cardiomyocytes with short telomeres (35, 152). Telomerase activity in human CSCs attenuates telomere shortening (152) but an average of 130bp of telomeric DNA is still lost after each cell division (13). Telomere-induced senescence in CSCs occurs when TL reaches around 1.5kb (153). As such, one overarching theme in the literature is that short telomeres force CSCs to undergo senescence and apoptosis. This, in turn, prevents cardiomyocyte replacement in the aging heart and leads to heart disease (35, 133, 148).

This loss of proliferative potential questions the true stem cell-like qualities of CSCs and some studies (38, 41, 57) have referred to cardiac cells positive for c-kit (the receptor of SCF (133)) as cardiac progenitor cells. Regardless of this limitation, however, cardiac cells with stem cell like qualities exist and can be extracted from the aged human heart and rapidly expanded in culture. In one study, average CSC TL decreased from 9.3 to 8.2 then 6.9kb *in vitro* after approximately 12, 18, and 27 population doublings respectively (13). Furthermore, CSCs extracted from aged hearts had telomeres 5.3-7.7kb (40), indicating that there is considerable growth reserve in the human heart throughout life. In another study, average

CSC TL post-MI was 7.5kb, more than three times longer than senescent cells, highlighting that viable CSCs with huge replicative potential are present even in the diseased heart (24). Nonetheless, age is a major predictor of telomere shortening, decreased telomerase activity, increased TIFs, p16 and p21 expression, which are all considered biomarkers of hCSC senescence (31). Of these, TL may be the best biomarker to identify CSCs capable of producing functional cells. Indeed, human CSCs with shortened telomeres produce new cells that inherit this trait and quickly acquire the senescent phenotype (70). Identifying the mechanisms that elongate cardiac telomeres could facilitate rejuvenation of the heart by producing functional, long-living cells *in vivo*. This could be achieved by harnessing and compounding the replicative potential of CSCs demonstrated in culture.

Enhancing cardiac stem cell proliferation

Although cardiomyocyte renewal by CSCs with short telomeres is destined to result in a phenotypically old heart (70), growth factors promoting mitosis can disrupt cellular senescence (133). Indeed, CSCs increase 1.9-fold in mice between 4 and 22 months of age (148) and around 3-fold from 4 to 28 months of age in rats (57). Importantly, however, the number of senescent cells also increases, resulting in fewer functional CSCs with age (148). This suggests that delaying cellular senescence in the heart would suffice to prevent age-associated cardiac decline. In this regard, the levels of cell death and replication are similar during aging in IGF-1 mice, which could explain the longer telomeres found in these animals throughout life (148). It was also demonstrated that IGF-1 activates telomerase in CSCs inducing them to divide via the P13K-Akt pathway, which provides an explanation for this increase in cardiomyocyte TL, proliferation, and lifespan (148). The phenotypic age of CSCs, like cardiomyocytes discussed above, can also be determined by the oxygen levels of the microenvironment. In this regard, hyperoxic CSCs do not suffer telomere attrition with age while normoxic CSCs and CMs suffered 46 and 43% decrease in TL respectively from three

to thirty months of age in mice (133). Once more, these differences in TL had functional consequences because 74% of hyperoxic CSCs could divide and replenish the heart whereas only 30% of normoxic CSCs could replicate (133). Importantly, however, exposure to hyperoxic conditions stimulated proliferation of CSCs, suggesting that improved oxygen supply could stimulate cardiomyocyte renewal (133). Similarly, overexpression of the cardioprotective kinase Pim-1 in primary 13-month old mouse CSCs, causes a transient increase in proliferation and TL (38). These factors provide possible mechanisms to temporarily increase CSC proliferation, which could allow sufficient repair after ischaemic death to prevent degenerative cardiac decline. Such treatments would also lower the risk of oncogenic complications that accompany the prevention of senescence and apoptosis (38).

Another option to preserve cardiac function with age is to stimulate only functional CSCs to divide. Indeed, CSCs with long telomeres remain throughout life and can produce phenotypically young cardiomyocytes (57). One study found that SCF reversed cardiac aging in mice by stimulating phenotypically young CSCs *in situ* (133). Importantly, newly formed cardiomyocytes following SCF treatment had 75% longer telomeres (133). To identify CSCs with maximal growth reserve, D'Amario and colleagues (39) examined CSCs from patients undergoing elective cardiac surgery for coronary artery disease for the presence of IGF-1, insulin-like growth factor-2, and angiotensin II (Ang II) and their receptors (39). The authors found that older CSCs had shorter telomeres and increased AngII and apoptosis whereas CSCs with IGF-1 receptors (IGF-1R) had the longest telomeres and highest proliferative capacity (39). Moreover, the IGF-1/IGF-1R pathway was found to promote CSC growth and survival as well as preserve TL (39), consistent with the increased TL and number of dividing CSCs found in IGF-1 mice (148). As a result, CSCs expressing only IGF-1R had a young phenotype with long telomeres and reduced apoptosis making them the ideal cells for cardiac repair (39). Finally, IGF-1 also inhibited AngII-induced cell death by 40% in old CSCs (39).

Collectively, these findings highlight not only the importance of growth factors and oxygen levels to preserve functional CSCs and cardiomyocytes but also their ability to reverse cardiac aging and disease.

CSCs can be isolated from human ventricle tissue, grown *in vitro*, and then injected back into the heart as a treatment for heart failure (13). This makes increasing CSC proliferation a promising way to facilitate cardiac regeneration after CSC infusion (38). Clinical trials on autologous CSC (one million cells) transplantation post-MI improved cardiac function and decreased infarct area by 30% one year after treatment (24) (CSC therapy is reviewed elsewhere (53)). To optimise CSC infusions, however, the transplanted cells will require a young phenotype of which the key molecular hallmark is long telomeres (70). Understanding CSC and cardiomyocyte preservation is also crucial to cancer therapies because cardiotoxicity is a side-effect of many antineoplastic drugs. The most notable of these are the anthracyclines such as doxorubicin (154), which causes telomere erosion through oxidative stress (87). In rats, doxorubicin administration caused heart disease and death and subsequent *in vitro* studies found this occurred through CSCs telomere erosion and apoptosis (41). Indeed, doxorubicin caused a 30% decrease in TL and the percentage of CSCs with telomeres <8kb increased more than four times despite the maintenance of telomerase activity (41). Another study found that Pim-1 decreased the toxic effects of doxorubicin in CSCs (38), highlighting the mechanistic involvement of telomeres in doxorubicin-induced heart failure. Interestingly, CSC transfusion can also promote the regeneration of cardiomyocytes *in vivo* and improve the survival of rats exposed to doxorubicin (41). This led to the suggestion of autologous CSCs being obtained and transfused before the administration of cardiotoxic drugs to prevent or manage subsequent heart failure (41). Interventions to maintain TL, however, may preserve heart function and obviate the need for dangerous, invasive, and costly surgical procedures.

In contrast to the body of research on CSCs, Senyo and colleagues (135) found that pre-existing cardiomyocytes are the primary source of new cells in both healthy and diseased mouse hearts. These researchers also discovered that cardiomyocyte proliferation increases adjacent injured myocardium despite an overall reduction in cardiomyocyte DNA synthesis with age (135). Similarly, Beltrami and colleagues (15) showed 4% of human cardiomyocytes were in proliferation (identified by Ki67 antigen expression) in areas adjacent infarcts and 1% at sites distant from injury. The notion of cardiomyocytes being responsible for the maintenance of the heart's cell population (and losing this ability with age) is a simple and powerful explanation for heart disease. In support of this suggestion, cardiomyocytes have shorter TLs than CSCs in mice (133); human fetal cardiomyocytes undergo a maximum of 25 population doublings *in vitro* (11) whereas CSCs extracted from aged hearts have significant growth reserve even after 27 population doublings (13); and, based on the telomere attrition of cultured CSCs, $1x10^{15}$ cells can be produced by CSCs from one individual before senescence is reached (13). Nonetheless, the ability of CSCs (6), as well as cardiomyocytes without detectable stem-cell qualities (123, 135), to divide is clear but the inability of the heart to regenerate with age, and particularly after injury, is even more obvious. Given this replicative potential *in vitro*, it is also possible that cardiomyocyte replacement *in vivo* is hindered by other factors, such as the deposition of collagen, and not the poor replicative potential of CSCs and cardiomyocytes. Whether cardiac cells can be induced to divide following injury, and protected from senescence and apoptosis to prevent disease, is a promising area of future research. To accomplish this, however, the factors influencing telomere-induced senescence and apoptosis will likely need to be elucidated.

Cardiac telomere length in animal models

The use of a mouse model with *Tert*-overexpression specific to the myocardium (12, 112) circumvents the limitations of systemic telomere dysfunction as seen in *Tert* and *Terc* knockout mice (discussed above). Nonetheless, all rodent models present with many crucial differences when compared to humans. For instance, cardiomyocytes isolated from mice have TLs varying from 14-58kb (131) compared to 1.5-15kb in humans (146). Furthermore, critical TL associated with cell senescence of human CSCs is around 1.5kb (153) whereas in mice it is approximately 14kb (131). The difference in TL between mice and humans could explain the development of the mdx/*Terc*$^{-/-}$ mouse as a model for the recessive cardiorespiratory disorder Duchenne Muscular Dystrophy (96). mdx mice have a nonsense mutation in the causal gene but are phenotypically healthy (27). When crossed with *Terc* knockouts, however, mdx mice exhibit cardiomyopathy and decreased lifespan as seen in human Duchenne Muscular Dystrophy (106). Interestingly, these phenotypes did not appear until the second generation, which again suggests that TL and not telomerase activity is responsible for the manifestation of the disease phenotype. In this regard, differences in cardiac telomere length or maintenance could explain the phenotypic heterogeneity of genetic cardiomyopathies in humans.

Ludlow and colleagues (86) utilised the CAST/Ei mouse, which has relatively short telomeres, similar to those in humans (61), but the C57BL/6 mice strain, which has relatively long telomeres (85), has still been used to study human heart disease (25, 133). Interestingly, telomeres in zebrafish and humans are of similar size (63), which could make zebrafish a good model of human cardiac disease in regards to telomere biology. In addition, the zebrafish heart can completely regenerate after injury (56) and this is accompanied by a transient increase in cardiomyocyte TL and proliferation (14). Furthermore, *Tert*$^{-/-}$ zebrafish

are unable to elongate telomeres or replenish their cardiac cell population after injury (14). These results highlight the involvement of telomerase and the importance of cardiomyocyte replacement but leave unanswered questions regarding the exact role telomeres have in the heart. Perhaps if telomere elongation could be inhibited in WT and *Tert*$^{-/-}$ zebrafish, the role TL plays in cardiac rejuvenation could be elucidated in this model. Despite similarities in human and zebrafish TL, however, the longer lifespan of humans suggests that more stringent telomere maintenance is required, and such mechanisms may not be present in short-lived animals. These interspecies differences highlight the need to validate animal findings in human models of disease.

Cardiac telomere attrition as a potential cause of heart failure

Another important question that has long been posited (136) but remains unknown (52) is whether telomere attrition is a cause or consequence of heart disease. As telomere length is cell-specific (43, 67, 86), the role of telomeres in disease development likely varies between different types of heart disease. Furthermore, uncovering these relationships will require the study of telomere regulation at the site/s of each specific cardiovascular pathology. Nevertheless, there are essentially only three possible explanations for the involvement of TL in any type of heart disease. Firstly, telomere attrition *causes* the disease; secondly, telomere shortening is a *consequence* of the disease; and thirdly, one or more *confounding* variables, such as oxidative stress and mitochondrial dysfunction, cause both telomere shortening and the disease. In the heart specifically, confounding variables pose particular challenges to demonstrating a cause and effect relationship between cardiac TL and function. For instance, as short TL is strongly associated with age (33, 60) and cardiac hypertrophy is a normal and necessary feature of development (50), cardiac TL and heart failure have the same primary risk factors. As discussed above, however, the dysregulated telomere maintenance observed in early-stage heart failure (80, 138, 153) and the propensity of telomerase-deficient mice to develop cardiac hypertrophy and subsequent heart failure (81), suggests insufficient telomere maintenance may *cause* cardiac hypertrophy and heart failure (Figure 4).

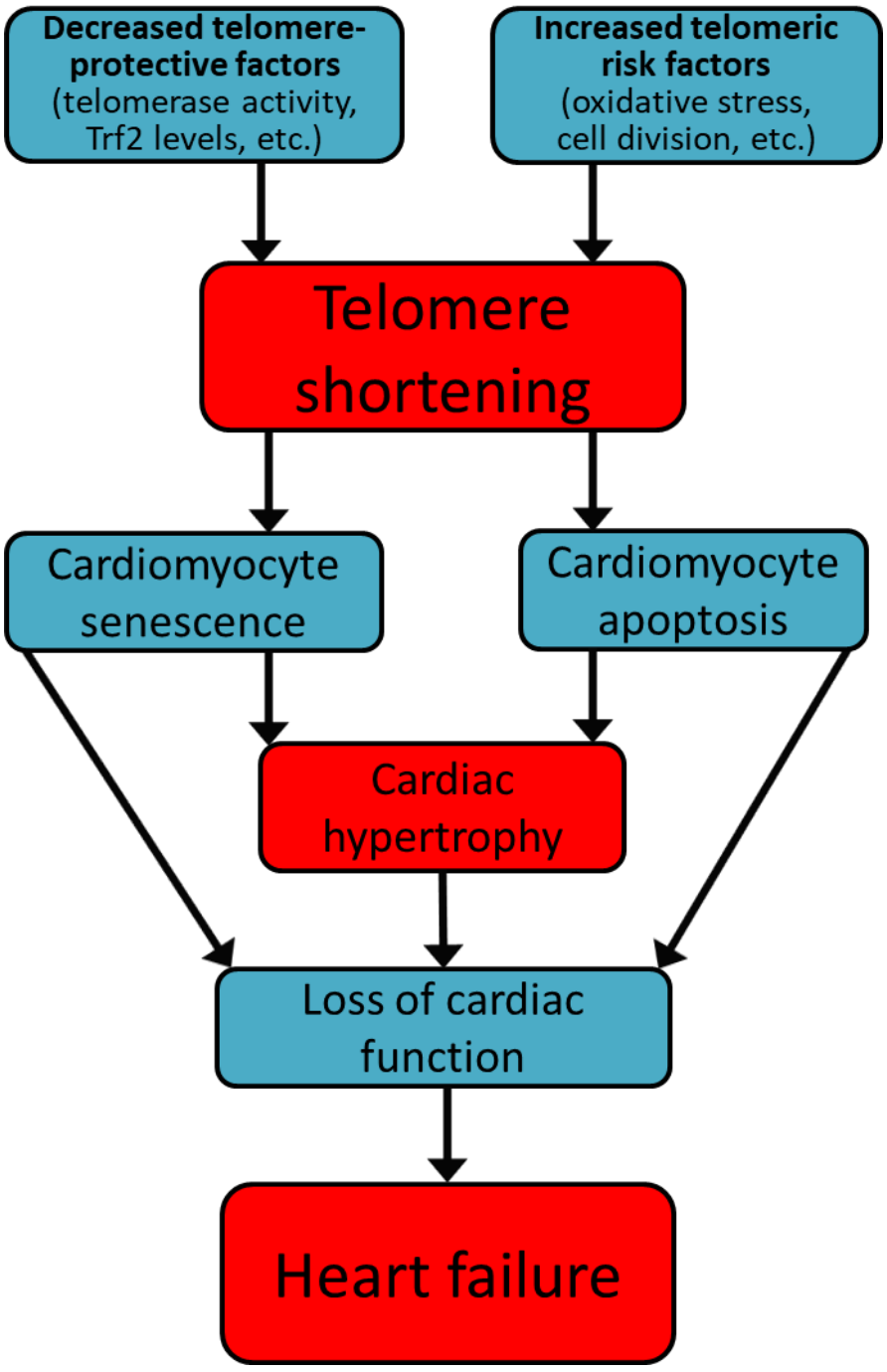

Figure 4. The possible causal involvement of telomeres in the pathophysiology of cardiac hypertrophy and heart failure. Trf2, telomere repeat-binding factor 2.

Conclusions

In conclusion, telomere attrition (113, 131, 148), as well as telomere-induced senescence (35, 148) and apoptosis (81, 166), is a consistent molecular feature of heart failure. Although it is possible that telomere shortening may only be associated with the onset and not the development of heart failure, this still implicates other telomeric variables, such as TERT and TRF2, in the early stages of disease. The interdependence and substantial redundancy of cellular activities has also made it difficult to identify causes of cardiac telomere attrition, aging, and disease. In this regard, cardiac hypertrophy could be used as a model of early-stage heart failure to determine the involvement of telomeric factors in disease progression. Elucidating the mechanisms of telomere maintenance in the heart holds promise to better prevent, diagnose, and treat cardiac dysfunction as well as potentiate regeneration after injury.

Aim and hypothesis

As discussed in my literature review, a significant body of research has demonstrated that TL is inversely associated with heart failure but the cause/s of short telomeres in failing hearts is unknown. Importantly, uncovering the involvement of telomere shortening in the pathophysiology of heart failure could provide novel targets for therapeutic and preventative interventions. As cardiac hypertrophy is the key antecedent to heart failure, the overall aim of this thesis was to determine the role of telomeres in the development of cardiac hypertrophy. It was hypothesised that decreases in telomere length and/or maintenance would increase cardiomyocyte size. This was formally tested with four experimental studies (Chapters 2-5).

Experimental design

Many of the animal models in which cardiac TL has been determined suffer from specific gene deletions or confounding comorbidities that could obscure the identification of factors involved in the development of cardiac hypertrophy. Similarly, many human studies have relied on measurement of TL from circulating leukocytes and it has not been shown if TL in circulating leukocytes is representative of the hypertrophic heart. To address these deficiencies in the literature, I first studied cardiac telomeres during the development of cardiac hypertrophy and progression to heart failure in the hypertrophic heart rat (HHR), a polygenetic model that suffers from primary cardiac hypertrophy independently of confounding diseases. I also measured TL in circulating leukocytes in the presence of established cardiac hypertrophy (Chapter 2). I then sought to determine if there were telomeric changes in adult intrauterine growth restricted (IUGR) rats, an environmental model of low birthweight and/or delayed postnatal growth leading to cardiac hypertrophy in adulthood. A subset of IUGR rats also underwent exercise training to investigate the influence of exercise on cardiac telomeric variables in the context of cardiac hypertrophy after growth restriction (Chapter 3).

Despite the utility of the HHR and IUGR as *in vivo* models of cardiac hypertrophy, important differences exist between rodents and humans in terms of TL, life span, and cardiomyocyte morphology. To determine the role of telomeres in human cardiac hypertrophy and if animal models are representative of this disease, human primary cardiomyocytes (HPCs) and rat immortalised cardiomyoblast (H9c2) cells were grown *in vitro*. I treated HPCs and H9c2 cells with five different hypertrophic agonists to increase cell size and then determined if this was accompanied by changes in telomere maintenance and/or length (Chapter 4). Conversely, I then disrupted telomere maintenance in HPCs using small

interfering RNAs (siRNAs) for *TERT* and *TRF2* as well as pharmacological inhibition of telomerase with the small molecule BIBR1532. Cell size was subsequently measured to determine if inhibiting telomere maintenance was sufficient to induce human cardiomyocyte hypertrophy (Chapter 5). The specific research questions addressed by each of these Chapters are listed below.

Research questions

Chapter 2

- Are there changes in cardiac and cardiomyocyte TL and/or telomeric gene expression and, if so, do they reflect the development of polygenic cardiac hypertrophy?
- Is leukocyte TL representative of cardiac and cardiomyocyte TL in polygenic cardiac hypertrophy?

Chapter 3

- Does cardiac hypertrophy induced by growth restriction affect cardiac TL?
- Can exercise training protect cardiac telomeres after growth restriction?

Chapter 4

- Can inducing cardiomyocyte hypertrophy cause telomere attrition and/or changes in telomeric gene expression in rat and/or human cells?
- Are there any changes inflammation, oxidative stress, and/or proliferation, that could also lead to telomere shortening in HPCs?

Chapter 5

- Can inhibiting telomere maintenance induce human cardiomyocyte hypertrophy?
- Does impaired telomere maintenance affect hypertrophic gene expression, proliferation, apoptosis, or oxidative stress in HPCs?

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Chapter 2

Telomere Length During Aging in a Rat Model of Polygenic Cardiac Hypertrophy

Abstract

Background and aims: Cardiac hypertrophy and short cardiomyocyte telomere length is associated with heart failure but there is no clear link. The aim of this study was to investigate telomere length, telomeric genes, and microRNAs in the heart before, during, and after the development of cardiac hypertrophy.

Methods and Results: The ontogeny of cardiac hypertrophy and subsequent heart failure was studied in the hypertrophic heart rat (HHR), a polygenic model of cardiac hypertrophy, and its control strain the normal heart rat (NHR). Ages used corresponded with the pathophysiological sequence beginning with fewer cardiomyocytes (2-days), the development and onset of cardiac hypertrophy (4- and 13-weeks) and progression to heart failure (38- and 52-weeks). Telomere length, telomerase activity, and expression of the telomeric microRNA miR-34a were determined using qPCR. Telomere length was longer in the HHR at 2-days and 38-weeks, shorter at 4- and 13-weeks, and not different at 52-weeks. Similarly, telomerase activity in the HHR was higher at 2-days, lower at 4- and 13-weeks, and not different at 38-weeks. miR-34a expression was a significant predictor of telomere length at all ages in both strains.

Discussion and conclusions: Longer telomeres in the HHR at 2-days may reflect fewer fetal and early postnatal cardiomyocyte divisions and explain the reduced number of cells in this model that predisposes to cardiac hypertrophy and heart failure. The sharp reduction in telomere length and telomerase activity in the HHR in early life may contribute to the development of cardiac hypertrophy but short cardiac telomeres were not present at the progression to heart failure.

Introduction

Telomeres, the specialized DNA-protein complexes located at the ends of eukaryotic chromosomes, are essential for genomic and cell viability. Telomere length and its age-dependent shortening is regarded as a marker of age-associated diseases in general and cardiovascular disease in particular (38). This is strongly supported by investigations showing that patients with atherosclerosis, coronary artery disease, chronic heart failure and stroke have shorter leukocyte telomere length (4, 13, 16, 43, 46). There are, however, some paradoxical findings in regards to telomere length and cardiac hypertrophy – one of the key indicators of target-organ damage in cardiovascular disease (24). For example, population-based studies have shown that cardiac mass and wall thickness are associated with longer, rather than shorter, telomeres (19, 44). This is difficult to explain since cardiac mass represents the time-averaged exposure to several cardiovascular risk factors which also decrease telomere length, such as age, inflammation, oxidative stress, hypertension, and obesity (6, 7, 23).

Less well studied is the importance of cardiac telomere dynamics to the developmental changes in cardiac mass leading to heart failure. Indeed, shorter telomeres at an early age can lead to impaired cell division, enhanced cardiomyocyte death and hypertrophy, all of which are concomitant with cardiac dysfunction and disease (22). Animal models have been be used to assess changes in telomeres (22), but in the case of cardiac hypertrophy this may be hindered by the confounding effects of hypertension (12, 30), and, hence, mask the genetic origin of this disease. This highlights the need to elucidate specific pathways that affect telomere length throughout the lifespan in the absence of increased blood pressure. Crucial to our understanding of the molecular mechanisms that underlie the development of cardiac hypertrophy and heart failure, and to the design of preventive and therapeutic regimens, are animal models that are bred to genetically develop cardiac hypertrophy leading to heart

failure. One such model is the hypertrophic heart rat (HHR), which develops cardiac hypertrophy independently of hypertension (15).

Given the established link between telomere dysfunction and cardiomyocyte proliferative defects (22), we postulated that cardiac telomere homeostasis would be perturbed in the HHR. To test this hypothesis, we first measured telomere length in the heart of the HHR at key ages corresponding to the pathophysiological sequence of fewer cardiomyocytes (2-days), cardiac hypertrophy (4- and 13-weeks) and subsequent progression to heart failure (38- and 52-weeks). In the search for the molecular basis of telomere regulation in the hypertrophic heart, we then measured changes in the telomere lengthening enzyme telomerase and quantified mRNA levels of its two subunits – telomerase reverse transcriptase (*Tert*) and telomerase RNA component (*Terc*). MicroRNAs (miRNAs), small non-coding RNA molecules which negatively regulate gene transcription by binding to the 3' untranslated region of their target mRNAs, have also emerged as key regulators of cardiac growth (40) and telomere length (3). As such, we also investigated whether changes in telomere length are due to alterations in the newly discovered telomeric regulator miR-34a (3, 35). Finally, to determine if leukocyte telomere length is representative of the hypertrophic heart, we measured telomeres in blood and corresponding cardiac tissue in 13-week old HHR and NHR.

Methods

Ethical clearance

The study was approved by the Animal Ethics Committees at the University of Melbourne and Deakin University, and ratified at Federation University Australia.

Animal models

The HHR is a polygenic model of cardiac hypertrophy that was developed by cross-breeding the spontaneously hypertensive rat, a model of hypertension and cardiac hypertrophy, and the Fischer 334 rat, which has normal blood pressure and a small heart. These strains were selectively interbred over 13 generations to create the HHR, which presents with pathological cardiac hypertrophy in the absence of a pressure load at maturity. The sister strain used as the control, the normal heart rat (NHR), has normal heart size and blood pressure (15). Compared to the NHR the HHR is born with a smaller heart containing fewer cardiomyocytes (33) but by 12 weeks of age presents with established cardiac hypertrophy which leads to premature death due to heart failure as early as 48 weeks of age (15, 33).

Samples and tissue collection

2-day old HHR (n=7, 5 females and 2 males) and NHR (n=9, 7 females and 2 males) were euthanized by decapitation. 4-week (n=10 HHR, 6 females and 4 males; and n=10 NHR, 4 females and 4 males), 13-week (n=9 HHR, 5 females and 4 males; and n=11 NHR, 6 females and 5 males), 38-week (n=9 HHR, 6 females and 3 males; and n=7 NHR, 4 females and 3 males), and 52-week old (n=12 HHR, 6 females and 6 males; and n=10 NHR, 5 females and 5 males) were euthanized with an overdose of pentobarbitone (Lethobarb). No more than one female and one male were used per litter. Hearts were immediately removed, and the left ventricle dissected from the atria. Cardiac weight index (CWI) was calculated from the total heart weight (mg) relative to total body weight (g) of each animal. Left ventricle tissue

samples were also collected from 52-week old HHR and NHR using the same method but heart weight and body weight data were not recorded. Finally, whole blood was collected in heparin tubes after cardiac puncture from 13-week old rats only as it was not available from other age groups. All tissues were first preserved in liquid nitrogen then later transferred to a –80^{o}C freezer.

Cardiomyocyte isolation

Cardiomyocytes were isolated from snap frozen tissue using a protocol adapted from a publication (28) and personal communication to Prof. Cris dos Remedios (University of Sydney). Briefly, cardiac tissue was sectioned into 300µm sections and stored at -20°C for 4 hours. We then added 1 mL of digestion buffer (PBS, 1mg/mL collagenase B, 1mg/ml collagenase D, and 30mM BDM, filtered using a 0.22µm filter) for every 300µm cardiac section and digested immediately for 20 minutes on an incubated rocking plate (37 °C, 99 RPM). The tubes containing the tissue where placed on ice and manually digested by pipetting the solution up and down repeatedly (~20 times with a 10ml sterile pipette). The 20-minute incubations and manual digestion steps were repeated 3 times. The solution was then passed through a 100µm mesh filter and increasing volumes (0.1, 0.2, 0.3 and 0.5) of RCGM media (Lonza) were added with 10 minutes rest intervals on ice. The cell pellet was collected by centrifugation at 200 RPM for 4 minutes at 4°C and this was used for the extractions described below.

DNA and RNA extraction

DNA from cardiac tissue, cardiomyocytes, and circulating leukocytes was extracted using the PureLink® Genomic Extraction kit (Life Technologies). RNA was extracted from cardiac tissue using the miRNeasy kit (Qiagen). All extractions adhered to the manufacturer's

instructions. Both RNA and DNA were quantified by spectrophotometry using a NanoDrop® ND-100 spectrophotometer (Thermo Scientific).

Telomere length

The telomere-repeat copy number (T) to single-copy gene number (S) ratio (T/S ratio) was determined by real-time quantitative PCR (qPCR). *Tel1* and *36B4* were used to reflect T and S, respectively, as previously described as previously described (5). Briefly, a 10ng template of extracted DNA was loaded in triplicate into a 384-well plate with SensiMix SYBR No-Rox (Bioline) and run in a Viia7 instrument (Life Technologies). Primer sequences and concentrations were: *Tel1* forward, GGTTTTTGAGGGTGAGGGTGAGGGTGAGGGT GAGGGT [300nM]; *Tel1* reverse, TCCCGACTATCCCTATCCCTATCCCTATCCCT ATCCCTA [300nM]; *36B4* forward, CAGCAACTGGGAAGGTGTAATCC [300nM]; and *36B4* reverse, CCCATTCTATCATCAACGGGTACAA [500nM]. Cycling conditions involved one hold step at 95^{o}C for ten minutes followed by 36 cycles of 95^{o}C for 15 seconds, 54^{o}C for 20 seconds, and 72^{o}C for 2 minutes. The square root of the average cycle threshold (CT) for *36B4* was then divided by the square root of the average CT for *Tel1* to yield the T/S ratio, a measure of telomere length.

Telomerase activity

Telomerase activity was measured using the TRAPeze® Kit RT Telomerase Detection Kit (Merck Millipore) and Platinum® Taq DNA Polymerase (Life Technologies) according to the manufacturer's instruction. Briefly, protein was extracted using CHAPS Lysis Buffer (Merck Millipore), and one µg of protein was added to each reaction. Samples were measured in duplicate, and telomerase activity was quantified relative to a standard curve by qPCR in a Viia7 instrument (Life Technologies). The activity of telomerase was presented as

the number of copies based on the standard curve supplied by the manufacturer. Telomerase activity was not determined in 52-week old rats.

miRNA and gene expression

The levels of *Tert* and *Terc*, both components of telomerase, the enzyme responsible for the elongation of telomeres, and protein phosphatase 1 regulatory subunit 10 (*Ppp1r10*, also known as and hereafter referred to as *Pnuts*) mRNA, and miR-34a were measured using qPCR. For mRNA levels, the first-strand complementary synthesis reaction was performed using the High Capacity cDNA Reverse Transcription Kit (Life Technologies). Primers were designed to flank exon-exon junctions using Primer3 (37) and NCBI tool Primer Blast. Amplification reactions used the SensiFast^{TM} SYBR Low-ROX Kit qPCR reagent (Bioline). The specificity of qPCRs was ensured by melting curve analysis and DNA gel electrophoresis (data not shown). For miR-34a expression, RNA was transformed to cDNA using the TaqMan® MicroRNA Reverse Transcription Kit for miRNA cDNA (Life Technologies). Amplification reactions used TaqMan assays and the TaqMan® Fast Advanced Master Mix (Life Technologies). For both mRNA and miRNA expression, all samples were run in duplicates in a Viia7 qPCR instrument (Life Technologies). The cycling conditions for gene expression involved a hold step of 95°C for ten minutes then 40 cycles of 95°C for 5 seconds, 58°C for 10 seconds, and 72°C for 10 seconds. For miRNA expression quantitation, samples were heated to 50°C for 2 minutes then 95°C for 20 seconds followed by 40 cycles of 95°C for 3 seconds and 60°C for 30 seconds. Glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*) was used as reference transcript for mRNA levels and the miRNAs RNU6, 4.5S and Sno87 for miRNA expression. Primers, probe assays, and conditions are listed in Table 1. Significance was assessed using $2^{-\Delta\Delta CT}$ for both mRNA and miRNA expression calculations and presented as the relative expression to allow inter- and intra-

group comparisons (39). miRNA and gene expression was not determined in 52-week old rats.

Table 1. Primers, probe assays and conditions used for gene and miRNA expression.

Gene name	Primer Sequence (5'→3')	Annealing temperature	Concentration	Product length
Gapdh	F: GGGGCTCTCTGCTCCTCCCTG	58ºC	200 nM	108 bp
	R: ACGGCCAAATCCGTTCACACC		200 nM	
Punts	F: CTCAAGCAGAACAACACAGCG	58ºC	200 nM	125 bp
	R: CTACTCTGGGAGCGGATGAC		200 nM	
Tert	F: AACTACGAGCGGACCAAACA	58ºC	200 nM	150 bp
	R: CCCTGTCACATCTGCCTTAAC		200 nM	
Terc	F: TGTTATAGCTGTGGGTTCTGTTCTT	58ºC	200 nM	91 bp
	R: CCGCTGCAGGTCTGAACTTT		200 nM	

Official microRNA symbol	TaqMan assay ID	Annealing temperature
rno-miR-34a	000426	60 ºC
4.5S	001716	60 ºC
Sno87	AF272707	60 ºC
RNU6B	001973	60 ºC

bp, base pairs; F: forward; R: reverse.

Statistical analyses

GraphPad PRISM (version 6) and SPSS (version 21) were used for graphing and statistical analyses, respectively. Data sets were tested for normal distribution using the D'Agostino & Pearson normality test and equal variances were analysed using the F test. An independent sample *t*-test, Welch's test, or Mann-Whitney test were used to compare data between the groups. Pearson's or Spearman's correlations were used to correlate telomere length, miR-34a and telomeric gene expression, and CWI data. Analyses of variance (ANOVA) using Tukey's multiple comparisons test was used to compare between ages within the same strain. Step-wise regression analyses were used to identify potential predictors of cardiac size and telomere length, with age and sex as independent variables (F-entry probability: 0.05, removal: 0.1). Significance was set at $P<0.05$.

Results

Cardiac size

Table 2 provides the characteristics of the samples used in this study. 2-day old HHR had significantly smaller hearts ($P<0.001$) but by 4 weeks of age, the HHR had significantly larger hearts than age-matched NHR ($P=0.023$). This early increase in CWI in the HHR was even more pronounced at 13-weeks ($P<0.001$) and continued until 38-weeks ($P=0.012$), the oldest age that was measured, validating the presence of cardiac hypertrophy in these animals (Table 2).

Table 2. Characteristics of HHR and NHR samples used in this study.

Age	**NHR**				**HHR**			
	n	Body weight (g)	Heart weight (mg)	CWI (mg/g)	n	Body weight (g)	Heart weight (mg)	CWI (mg/g)
2-day	9	5.9±0.2	44.1±2.0	7.4±0.1	7	5.3±0.3	34.3±2.4**	6.4±0.2***
4-week	10	43.2±1.6	247.2± 8.1	5.7±0.1	10	46.5±3.0	293.0±13.5**	6.4±0.3*
13-week	11	205.2±15.9	697.0±44.8	3.4± 0.1	9	186.2±13.0	890.8±46.8**	4.9±0.3***
38-week	9	355.0±35.7	1188.6±83.0	3.4±0.1	7	253.1±20.5*	1038.5±108.8	4.5±0.5*

CWI, cardiac weight index. Values are represented as mean ± standard error of mean. * indicates $P<0.05$, ** $P<0.01$, *** $P<0.001$.

Cardiac and cardiomyocyte telomere length

There was a positive correlation between telomere length in isolated cardiomyocytes and cardiac tissue in both strains at all ages (r=0.295, P=0.017). Cardiac telomere length was significantly longer in 2-day old HHR (P=0.023) but shorter in 4-week (P=0.012) and 13-week old HHR (P=0.024) compared to age-matched NHR (Figure 1A). In 38-week old HHR, cardiac telomere length was significantly longer than the NHR (P=0.009) but there were no differences in telomere length between the strains at 52 weeks of age (P=0.261) (Figure 1A). When we compared cardiac telomere length within strains, 2-day old HHR had significantly longer telomeres than all other ages (P<0.05) and 52-week old HHR had significantly shorter telomeres than all other ages (P<0.001) (Figure 1B). In the NHR, telomeres were significantly shorter at 38-weeks (all P<0.05) and 52-weeks (all P<0.001) when each was compared to all younger ages (Figure 1C).

Circulating leukocyte telomere length

To investigate whether telomere length in circulating leukocytes is representative of the heart, we measured telomere length in circulating leukocytes and matching cardiac tissue from 13-week old NHR and HHR. As with cardiac telomere length, leukocyte telomere length was significantly shorter in 13-week old HHR compared to age-matched NHR (P=0.048) (Figure 1D). Moreover, there was a positive correlation between cardiac and leukocyte telomere length (r=0.448, P=0.031) (Figure 1E) but this correlation was driven by the HHR (r=0.81, P=0.026) as we did not detect a significant correlation in the NHR (r=0.18, P=0.605).

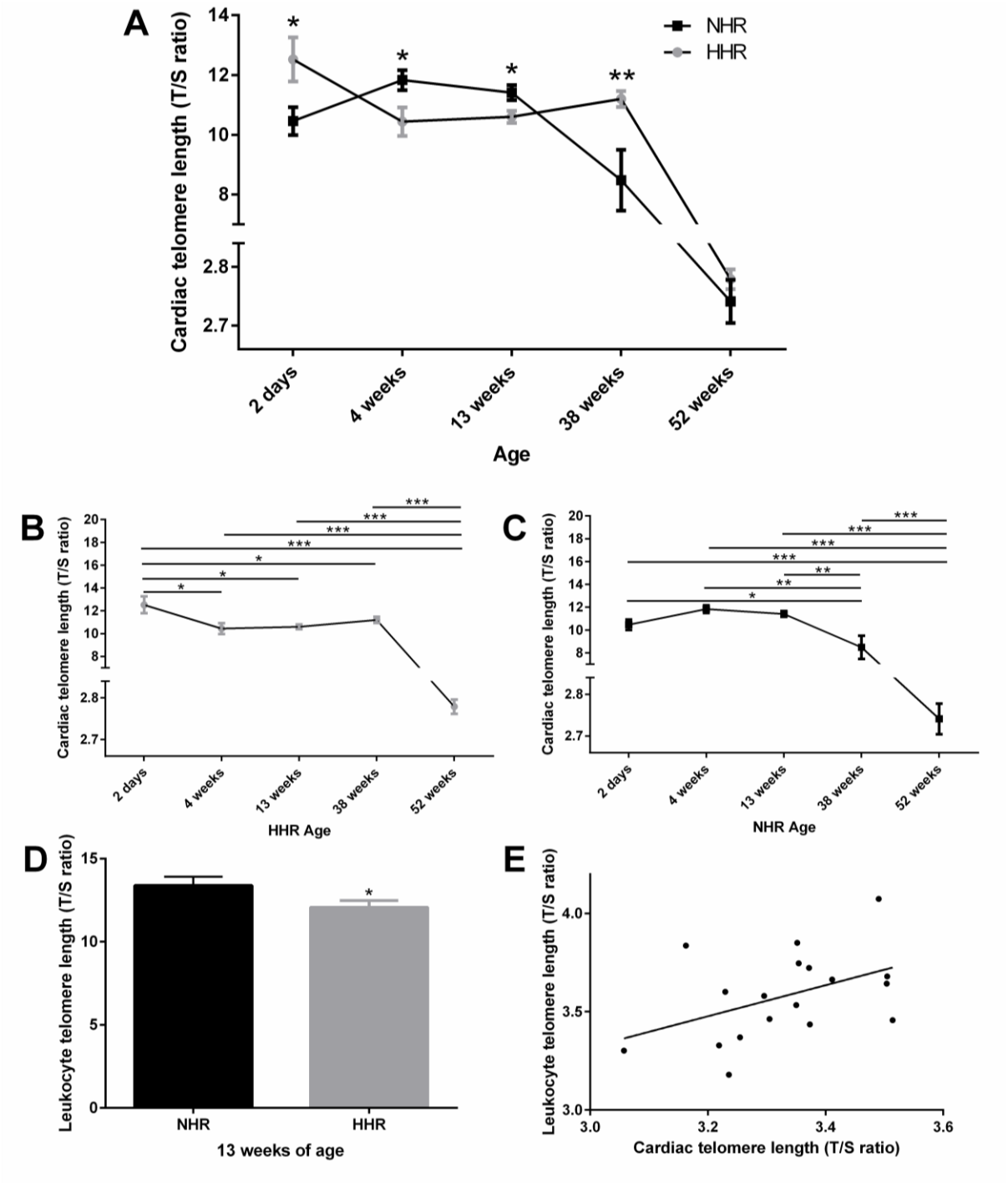

Figure 1. Telomere length. (A) At 2-days the HHR has significantly longer cardiac telomere compared to the NHR. In 4- and 13-week old animals, the HHR has shorter telomeres than age-matched NHRs. At 38-weeks of age, telomeres are again longer in the HHR compared to the NHR. (B) The shortening of telomeres in the HHR happened early in life compared to (C) the NHR where telomere shortening occurred in late adulthood. (D) Circulating leukocyte telomere length was shorter in 13-week old HHR compared to age-matched NHR. (E) Circulating leukocyte telomere length is correlated to cardiac telomere length in 13-week old animals. The appropriate independent-sample *t*-tests were used for (A) and (D), ANOVAs for (B) and (C), and Pearson's correlation for (E). Graphs represent mean, error bars represent standard error of the mean. * indicates $P<0.05$, ** $P<0.01$, *** $P<0.001$.

Telomerase activity

In accordance with cardiac telomere length, telomerase activity was higher in 2-day old (P<0.001) and lower in 4-week old HHR (P=0.005) (Figure 2A). At 13- and 38-weeks of age, however, we did not discover significant difference in telomerase activity between strains (both P>0.05) (Figure 2A). Within strains, both 2-day old HHR (Figure 2B) and NHR (Figure 2C) had significantly higher telomerase activity than all other ages (all P<0.01). Moreover, telomerase activity was correlated to telomere length at all ages (r=0.27, P=0.035), especially in the NHR (r=0.51, P=0.005). Telomerase activity was not measured at 52 weeks of age as there was no change in cardiac telomere length in this group.

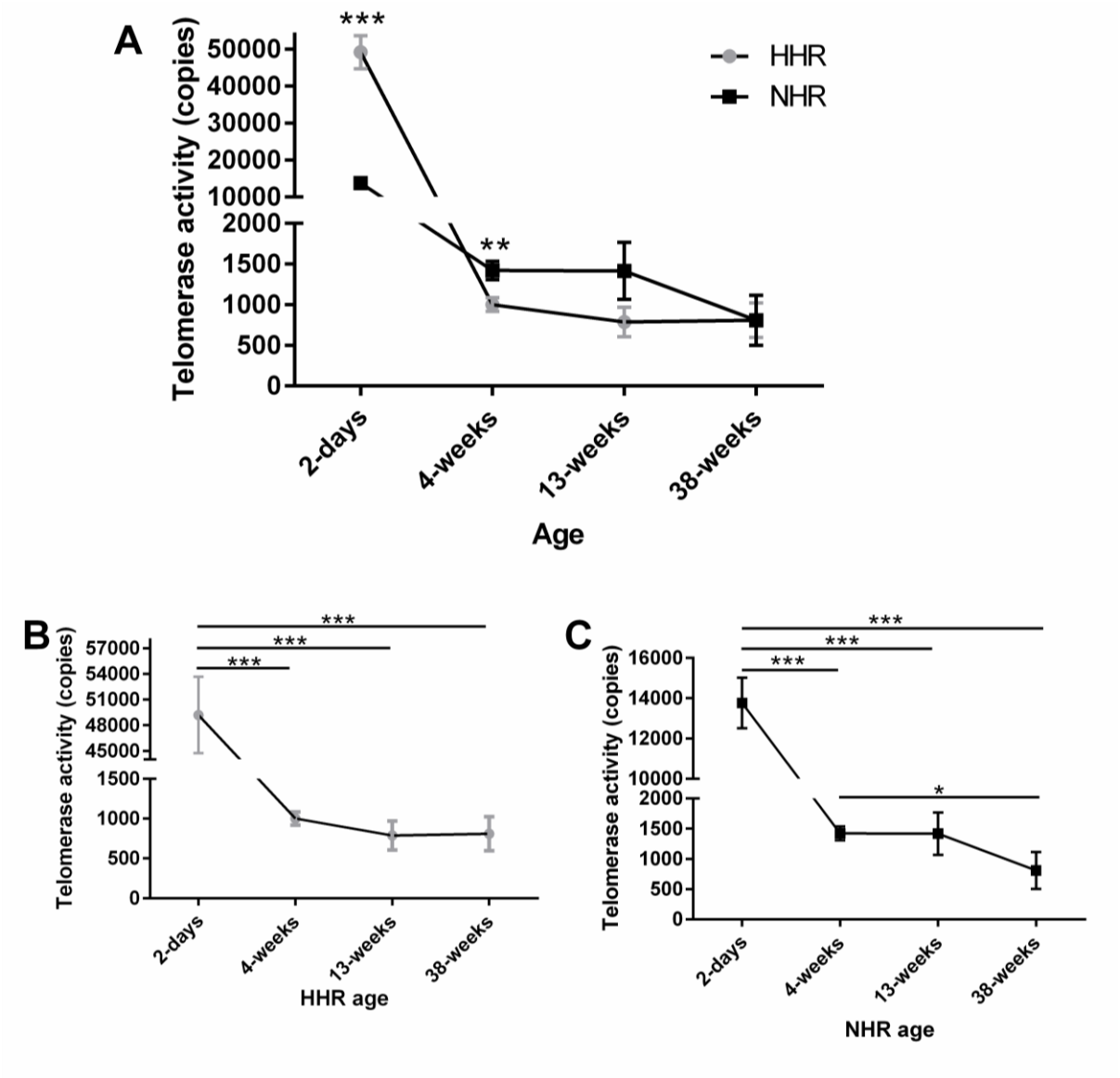

Figure 2. Telomerase activity. (A) Telomerase activity is higher at 2-days and lower at 4- in the HHR while there is no difference between strains at 13- or 38-weeks. (B) In the HHR, telomerase activity is higher at 2-days compared to all other ages. (C) In the NHR, telomerase activity is also higher at 2-days compared to all other ages along with a second significant decrease between 4- and 38-wees of age. The appropriate independent-sample *t*-tests were used for (A), ANOVAs for (B) and (C). Graphs represent mean, error bars represent standard error of the mean. * indicates $P<0.05$, ** $P<0.01$, *** $P<0.001$.

Tert and *Terc* expression

Tert expression was up-regulated at 2-days (P=0.021) and 38-weeks (P<0.001) but down-regulated at 4-weeks (P<0.001) in the HHR compared to age-matched NHR. There was also a tendency for *Tert* mRNA levels to be higher at 13-weeks of age, but this did not reach significance (P=0.066) (Figure 3A). In accordance with telomere length, *Tert* mRNA levels in the HHR were significantly lower at 4- and 13-weeks compared to 2–days and 38-weeks of age (all P<0.001) (Figure 3B). In the NHR, *Tert* expression was significantly lower later in life, at 13- and 38-weeks, when compared to younger 2-day and 4-week old rats (all P<0.001) (Figure 3C).

Terc mRNA levels did not significantly differ between the HHR and NHR at 2-days or 13-weeks of age (both P>0.05). At 4-weeks, however, the HHR had significantly lower *Terc* expression (P=0.046) while at 38-weeks the HHR had significantly higher *Terc* mRNA levels (P=0.018) (Figure 3D). Within the HHR strain, *Terc* expression was significantly higher at 38-weeks of age compared to all other ages (P<0.01) (Figure 3E). In the NHR, *Terc* mRNA levels were significantly higher at 4-weeks compared to 2-days and 13-weeks (both P<0.001) and 38-weeks compared to 2-days and 13-weeks (both P<0.01) (Figure 3F).

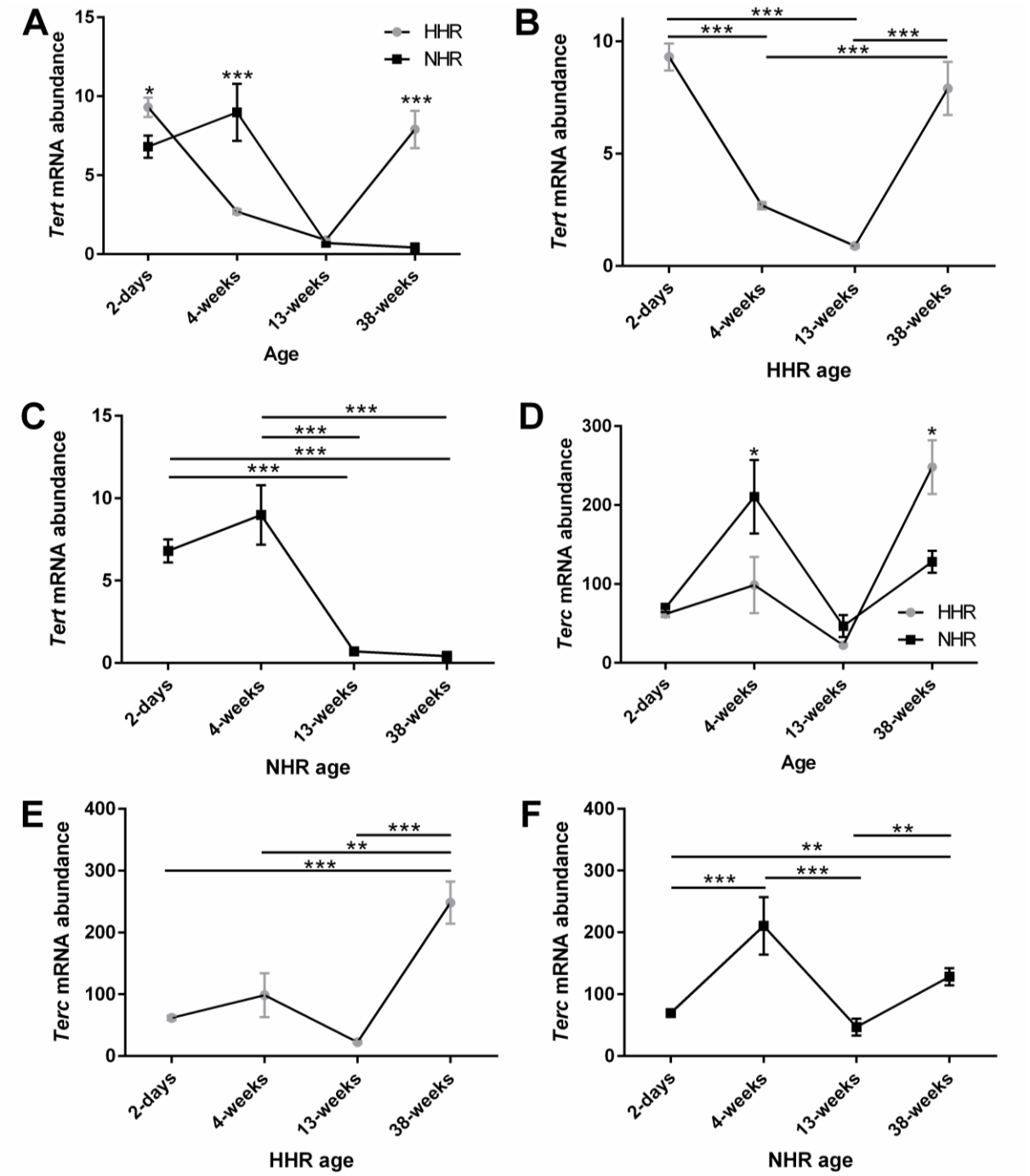

Figure 3. *Tert* and *Terc* expression. (A) *Tert* mRNA levels were significantly higher at 2-days and 38-weeks but lower at 4-weeks in the HHR compared to the NHR. (B) In the HHR, *Tert* expression is lower and 4- and 13-weeks when compared to 2-day and 38-week old animals. (C) In the NHR, *Tert* expression is higher in 2-days and 4-weeks when compared to 13- and 38-weeks. (D) *Terc* mRNA levels are significantly higher in the NHR at 4-weeks and the HHR at 38-weeks with no differences at 2-days or 13-weeks. (E) In the HHR, *Terc* expression was higher at 38-weeks than all other ages. (F) In the NHR, *Terc* expression was higher at 4-weeks when compared to 2-days and 13-weeks and at 38-weeks when compared to 13-weeks and 2-days. The appropriate independent-sample *t*-tests were used for (A) and (D), ANOVAs for (B), (C), (E), and (F). Graphs represent mean, error bars represent standard error of the mean. * indicates $P<0.05$, ** $P<0.01$, *** $P<0.001$. Tert, telomerase reverse transcriptase; Terc, telomerase RNA component.

miR-34a and *Pnuts* expression

The miRNA miR-34a was significantly over-expressed in HHR compared to the NHR at 2-days (P=0.004), 4-weeks (P=0.024) and 13-weeks (P<0.001), but down-regulated at 38-weeks (P=0.019) of age (Figure 4A). In the HHR, miR-34a had the highest expression at 38-weeks of age compared to other ages (P<0.001), and was also higher at 13-weeks compared to 4- weeks and 2-days (both P<0.05) (Figure 4B). In the NHR, miR-34a was also upregulated at 38-weeks compared to all other ages (P<0.001), and at 13-weeks compared to 2-days (P<0.001) (Figure 4C).

Pnuts was previously described as a target of miR-34a in the mouse heart and plays an anti-apoptotic role in cardiomyocytes (3). Since miR-34a was over-expressed in the HHR, we measured *Pnuts* mRNA levels and found it was significantly down-regulated in the HHR at 4-weeks (P<0.001) but over-expressed at 38-weeks (P<0.001) (Figure 4D). Within the HHR, *Pnuts* mRNA levels were significantly lower at 4- (P<0.05) and 13-weeks (P<0.05) when compared to all other ages (Figure 4E). In the NHR, *Pnuts* expression was significantly higher at 2-days (P<0.05) and 4-weeks (P<0.05) when compared to all other ages (Figure 4F). *Pnuts* mRNA levels were borderline negatively correlated with miR-34a levels in all ages besides 38-weeks (2-days r=–0.38, P=0.067; 4-weeks r=–0.32, P=0.113; and 13-weeks r=–0.35, P=0.067).

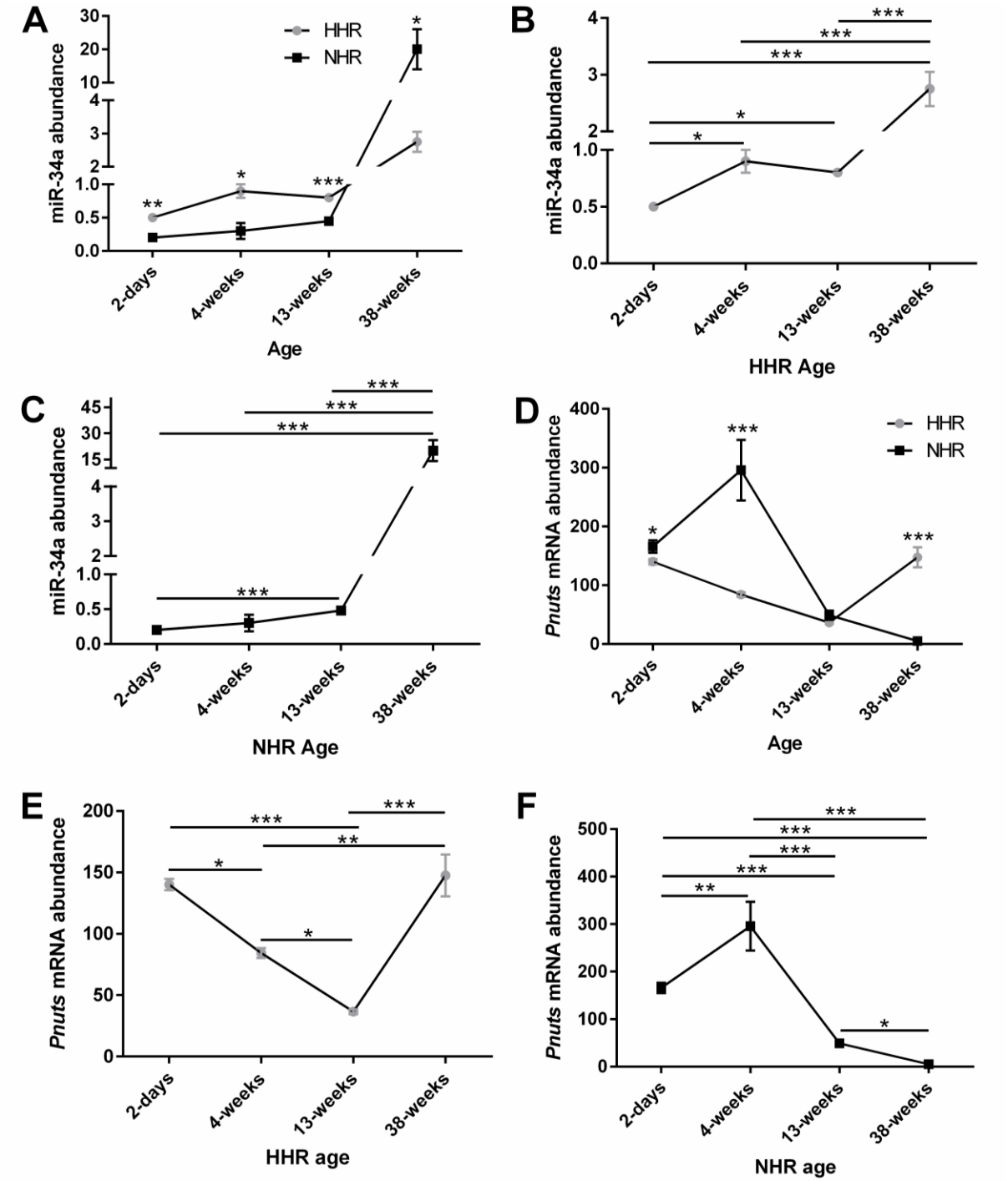

Figure 4. miR-34a and *Pnuts* expression. (A) miR-34a was upregulated in the HHR at all ages besides 38-weeks when it was lower than in the NHR. In both the HHR (B) and NHR (C) miR-34a expression at 4-weeks was significantly higher than all other ages and 13-weeks was higher compared to 2-days. (D) *Pnuts* mRNA levels were lower at 2-days and 4-weeks and higher at 38-weeks in the HHR compared to the NHR. In the HHR (E), *Pnuts* expression was significantly lower at 4- and 13-weeks compared to 2-days and 38-weeks while in the NHR (F) *Pnuts* was higher at 2-days and 4-weeks compared to 13- and 38-weeks. The appropriate independent-sample *t*-tests were used for (A) and (D), ANOVAs for (B), (C), (E), and (F). Graphs represent mean, error bars represent standard error of the mean. * indicates $P<0.05$, ** $P<0.01$, *** $P<0.001$. miR-34a, microRNA-34a; *Pnuts*, protein phosphatase 1 regulatory subunit 10.

Predictors of cardiac weight index and cardiac telomere length

In a step-wise regression analysis, cardiac telomere length was a significant negative predictor of CWI in both strains at all ages (Table 3). According to the standardised β coefficients, however, age had the largest effect on CWI, while the contribution of telomere length was similar to strain and sex (Table 3). In another step-wise regression analysis, *Tert* mRNA and miR-34a levels were the only molecular determinants of cardiac telomere length in all samples (Table 4).

Table 3. Significant predictors of cardiac weight index.

Variable	β±S.E.	Standardised β	95% C.I.	*P*-value
Age in weeks	–0.20±0.02	–0.80	–0.24 to –0.16	<0.0001
Female compared to male	–0.54±0.22	–0.18	–0.97 to –0.11	0.016
HHR compared to NHR	0.44±0.20	0.15	0.03 to 0.84	0.038
Cardiac telomere length	–1.03±0.50	–0.15	–2.03 to –0.03	0.043

C.I., confidence interval; S.E., standard error. Results are from a step-wise regression analyses used to determine predictors of cardiac weight index. Covariates included cardiac telomere length, age, sex (female compared to male), and strain (HHR compared to NHR). n=44 female and n=27 male rats. F-entry probability: 0.05, removal: 0.1.

Table 4. Significant predictors of cardiac telomere length.

Variable	β±S.E.	Standardised β	95% C.I.	*P*-value
miR-34a	–0.02±0.003	–0.62	0.03 to 0.84	0.038
Tert mRNA	0.015±0.50	0.22	–2.03 to –0.03	0.043

C.I., confidence interval; S.E., standard error. Results are from a step-wise regression analyses used to determine predictors of cardiac telomere length. Covariates included miR-34a, *Pnuts*, *Terc*, *Tert*, telomerase activity and strain. F-entry probability: 0.05, removal: 0.1.

Discussion

Dissecting the effect of telomere length on cardiac hypertrophy and heart failure has been hindered by the difficulty of obtaining human cardiac tissue and by the lack of animal models that are not affected by pressure overload. Here we set out to investigate the changes in telomere length in a normotensive model, the HHR, during the development of polygenic cardiac hypertrophy and progression to heart failure. We found that the HHR was born with longer telomeres, higher telomerase activity, and smaller hearts compared to the NHR. This was followed by accelerated telomere shortening that paralleled the development of cardiac hypertrophy. Alterations in telomere length were linked to dysregulation of the telomeric regulators *Tert* and miR-34a at all ages. Other molecular changes associated with telomere length at some ages included fluctuations in telomerase activity as well as *Pnuts* and *Terc* *m*RNA levels. This highlights the importance of these variables to cardiac telomere length and, hence, the dynamic regulation of cardiomyocyte growth and function (summarised in Figure 5).

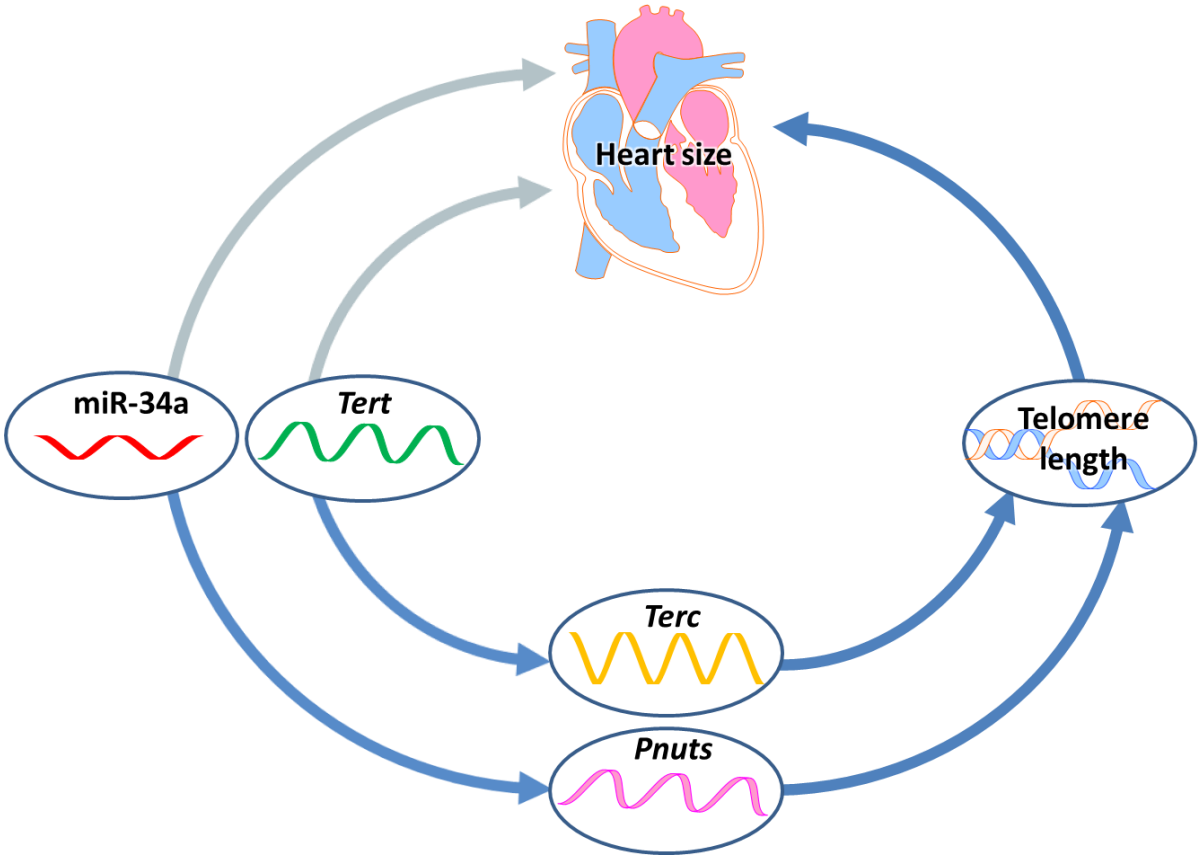

Figure 5. A simplified diagram of molecular mechanisms involved in regulating cardiac telomere length and heart size. Tert synergises with Terc to form telomerase, while miR-34a acts through regulating levels of *Pnuts* to maintain telomere length and heart size (blue arrows). Both Tert and miR-34a may also have hypertrophic effects on the heart through other pathways (grey arrows) such as increased proliferation (31) and apoptosis (3). Tert, telomerase reverse transcriptase; Terc, telomerase RNA component; miR-34a, microRNA-34a; *Pnuts*, protein phosphatase 1 regulatory subunit 10.

The concept of being born smaller and having longer telomeres, as observed in the HHR, is consistent with results found in young men born small due to intra-uterine growth restriction (20). The longer telomeres in 2-day old HHR could, at least in part, be due to fewer cell divisions in the heart during embryogenesis. This would also explain the reduced complement of cardiomyocytes previously reported in the HHR (33). Furthermore, recent studies in mice have shown that cardiomyocytes proliferate until post-natal day 7 (34), which is followed by another proliferative burst in the third week of life (29). In this regard, it is possible that cardiomyocytes in the HHR may be forced to divide more in postnatal life to compensate for their reduced number, which would contribute to the accelerated telomere attrition between 2-days and 4-weeks of age. Moreover, cardiomyocytes produced from cells with short telomeres inherit this defect and quickly acquire the senescent phenotype (17), limiting these cells to hypertrophic growth and predisposing to heart failure (17, 36). When combined with the sharp reduction in telomere length observed in the HHR in early life, this suggests telomere shortening could play a causal role in the development of cardiac hypertrophy.

In the current study, we also found significantly higher telomerase activity in neonatal HHR which may also contribute to the longer telomeres found in these animals. Indeed, telomerase activity decreased by around 60 times between 2-days and 38-weeks of age in the HHR, while in the NHR this was less pronounced with only around 17 times less telomerase activity at 38-weeks. Interestingly, telomerase also promotes cell viability independently of telomere length (14, 41, 45), suggesting it may contribute to cardiac hypertrophy through non-telomeric pathways. Indeed, the rapid decrease in telomerase activity and expression of its catalytic component *Tert* at critical stages of cardiac growth in the HHR, implicates these variables in the development of cardiac hypertrophy as drivers of telomere length and potentially through other mechanisms.

Another key finding of this study was the over-expression of miR-34a in the HHR at several ages. During normal development, over-expression of miR-34a was only previously reported in the aged heart, a phenomenon which leads to cell death (3). This suggests that the up-regulation of this miRNA early in life could also be involved in the reduced complement of cardiomyocytes in the HHR (33), thereby stimulating compensatory hypertrophy in the remaining cells (17, 36). Indeed, therapies targeting miR-34a or the miR-34 family by Bernardo and colleagues (1, 2) were shown to have beneficial effects in the diseased heart. Furthermore, we found that *Pnuts* mRNA, a target of miR-34a, was down-regulated in the HHR at the onset of hypertrophy and this gene is known to have an important role in telomere maintenance by directly interacting with non-redundant telomeric protein telomere repeat-binding factor 2 (Trf2) (18, 32). Indeed in primary rat cardiomyocytes, decreased expression of *Trf2* causes accelerated telomere attrition and increased apoptosis (32). Furthermore, Trf2 protects against apoptosis even in non-dividing cells (21), highlighting the importance of this pathway in the heart.

Consistent with the documented positive correlation between cardiac mass and longer leukocyte telomere length in humans (19, 44), 38-week old HHR had significantly longer telomeres than the age-matched controls. Importantly, however, our data indicate that telomere length in the HHR does not show significant increases with age. Instead, telomeres were longer at birth, and then become shorter early in development, with no significant changes observed thereafter. Indeed, cardiomyocyte division in adulthood has been shown to occur at a slower pace, accounting for only a fraction of the telomere attrition observed throughout the lifespan (34). This phenomenon was also observed in the NHR, where telomere shortening happened only later in life. These findings suggest that the various mechanisms involved in telomere maintenance and their relative importance may differ between normal and hypertrophic hearts. In this regard, we showed a significant correlation

between leukocyte and cardiac telomere length in the HHR but not in the NHR. Although some research has shown synchronous telomere shortening between tissues (8), our findings combined with others (11, 26) suggest that telomere length in circulating leukocytes does not reliably reflect telomere measurements in the heart.

The longer telomere length in the presence of cardiac hypertrophy may seem paradoxical, but the adaptive response of the heart to stress is a ubiquitous theme in the literature. For instance, telomerase activity in dogs was increased at the onset and further again at the progression of ventricular dysfunction (25). Similar results have been found in human hearts (42). While compensatory increases in telomerase activity at the development of cardiac hypertrophy could explain the longer telomeres in the HHR at 38-weeks and in humans with cardiac hypertrophy (19, 44), this was not detected at the ages measured in the present study. This points to the involvement of other protective mechanisms, such as *Terc* and *Pnuts* mRNA levels, which were upregulated in older HHRs.

The increase *Terc* and *Pnuts* expression could also explain, at least in part, the surprising finding that short telomeres were not present in the HHR at 52-weeks of age. It is possible, however, as heart and body weight data were not available, that this cohort did not have cardiac hypertrophy compared to the NHR. Indeed, cardiac telomere length was significantly shorter than all other ages at 52-weeks in the NHR as well as the HHR. In this regard, the negative regulator of telomere length and proapoptotic miR34-a (3) was upregulated at 38-weeks in the NHR which could contribute to the reduction in telomere length at 52-weeks of age. Furthermore, all animals in this study were euthanised and, as such, the HHRs had outlived some members of previous cohorts which have died from heart failure as early as 48 weeks of age (33). In this regard, we would expect significantly shorter telomeres in post-mortem examinations of diseased hearts, as previously reported (32).

Although the use of qPCR to measure relative telomere length is widely used in the literature (6, 7, 10), the lack of cell-specific or critically-short telomere measurements needs to be acknowledged as a limitation. Previous studies, however, have shown that this method is reliable and the results are highly comparable to other techniques, such as mean terminal restriction fragment (TRF) length (5) and single telomere length analysis (STELA) (27). Moreover, we have previously shown that telomere length varies according to the method of DNA extraction (9). In the present study, all samples were collected, processed, and extracted using the same method, and samples were run in the same qPCR plate to minimise variation. Therefore, we believe the results presented here are highly robust. Irrespectively, however, it would be still useful to validate these findings in other animal and human models of cardiac hypertrophy.

Findings from this study suggest that telomeres in cardiomyocytes and cardiac tissue play an important role in the development of cardiac hypertrophy, independently of hypertension. We found accelerated telomere shortening, decreased telomerase activity, and lower *Tert* mRNA levels in the HHR early in life which paralleled increases in cardiac mass. Although the NHR also suffers from age-associated changes in telomeric variables and cardiac mass, these changes are more pronounced and present early in life in the HHR. As such, these telomeric regulators could be targeted for diagnostic and therapeutic use to identify and treat early-stage cardiac hypertrophy, thereby preventing the development of degenerative heart failure.

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Chapter 3

Cardiac Telomere Length in Growth-restricted and Exercise-trained Rats

Abstract

Background and aims: Fetal and postnatal growth restriction predisposes to cardiac hypertrophy and subsequent cardiovascular disease in adulthood. Telomeres are repetitive DNA-protein structures that protect chromosome ends, and the loss of these repeats (a reduction in telomere length) is associated with cardiac hypertrophy. As exercise preserves telomere length and heart function, the aim of this study was to determine the effects of growth restriction and exercise training on cardiac telomere length and telomeric genes.

Methods and results: Pregnant Wistar Kyoto rats underwent bilateral uterine vessel ligation to induce uteroplacental insufficiency and fetal growth restriction ('Restricted'). Sham operated rats had either intact litters ('Control') or their litters reduced to five pups with slowed postnatal growth ('Reduced'). Control, Restricted, and Reduced male rats were then randomly assigned to Sedentary, Early exercise (5-9 weeks of age) or Late exercise (20-24 weeks of age) groups. Hearts were excised at 24 weeks of age for telomere length and gene expression measurements by qPCR. Growth restriction shortened cardiac telomere length ($P<0.001$) but this was rescued by early exercise ($P<0.001$). Early and Late exercise increased cardiac weight index ($P<0.001$) but neither this nor telomere length was associated with expression of the telomeric genes *Tert*, *Terc*, *Trf2*, *Pnuts*, or *Sirt1*.

Discussion and conclusions: Growth restriction shortens cardiac telomere length, reflecting the cardiac pathologies associated with low birth weight. Exercise in early life may offer long-term protective effects on cardiac telomere length which could help prevent adverse cardiac events in later life.

Introduction

Cardiac hypertrophy (also known as left ventricular hypertrophy) is an abnormal increase in cardiac mass (23) and the most potent risk factor for cardiovascular disease after old age (32). Although reversible in its early stages, cardiac hypertrophy is asymptomatic and typically becomes degenerative before diagnosis (22), leading progressively to heart failure (57). This highlights the need to identify at-risk individuals and implement preventative treatments as well as reverse cardiac decline in established cardiovascular disease.

With the exception of rare congenital heart defects and genetic cardiomyopathies, the earliest risk factors for developing heart disease are low birth weight (6, 24, 54) and reduced growth in the postnatal period (7, 9). In rats, uteroplacental insufficiency results in low birth weight ('Restricted') and a reduction in litter size slows postnatal growth ('Reduced') (49). Despite a normal heart to body weight ratio at birth (49), both Restricted and Reduced rats have fewer cardiomyocytes (10, 45) and develop established cardiac hypertrophy by 22 weeks of age (66), making them an ideal model to study the epigenetic causes of cardiac hypertrophy.

One key preventative factor for cardiovascular disease is exercise (4, 19) which induces an adaptive increase in cardiac mass known as *physiological* cardiac hypertrophy (25, 43). We recently reported that a 4-week endurance exercise treatment at 5- or 20-weeks of age increases heart mass at 24-weeks of age in both Restricted and Reduced male rats (60). These findings suggest that exercise may help remodel the cardiac hypertrophy commonly detected in growth-restricted individuals (6, 7, 9, 24, 54) to better reflect the physiological type. This, however, remains to be characterised and the molecular mechanisms involved in physiological changes in the heart are poorly understood.

Telomeres are non-coding TTAGGG repeats that complex with proteins to protect chromosome ends from degradation (12, 18). A loss of telomeric repeats (a reduction in telomere length) and protective proteins, such as telomere repeat-binding factor 2 (Trf2), causes cellular senescence and apoptosis which can lead to cell loss and organ failure (3, 11), as reported in heart disease (16, 51). Failing hearts also have decreased expression of the telomere-lengthening enzyme telomerase and associated telomeric genes (41, 50), particularly Trf2 (51). In contrast, endurance exercise can preserve these pathways and telomere length (33, 65), suggesting that telomeric gene expression and telomere length may be integral to the development of physiological cardiac hypertrophy (14).

Despite mounting evidence that telomere length (16, 39, 51) and exercise (20, 27, 28, 42, 65) are positively associated with cardiac function, these relationships are not well defined. The aims of this study were to determine whether telomere length and telomeric gene expression are altered in Reduced and Restricted rats and if this can be influenced by Early (5-9 weeks of age) and Late (20-24 weeks of age) endurance exercise training. It was hypothesised that Restricted and Reduced rats would have shorter cardiac telomeres and reduced telomeric gene expression and that this would be attenuated with exercise.

Methods

Ethical approval

This study was approved by the University of Melbourne Animal Experimentation Ethics Sub-committee, ratified at Federation University Australia, and conducted in accordance with accepted standards of humane animal care.

Animal models

The animals in this study have been described previously (34, 35, 60). Briefly, Wistar Kyoto rats aged 9-13 weeks were obtained for breeding purposes from the Australian Resource Centre (Murdoch, WA, Australia). Rats were then housed in a 12-hour light-dark cycle with *ad libitum* standard chow and water.

To induce uteroplacental insufficiency, the primary cause of low birthweight in the developed world (8), pregnant rats underwent bilateral uterine vessel ligation surgery to create the 'Restricted' group or a sham operation to create the 'Control' group. Both surgeries were performed on day 18 of gestation (term 22 days) and used intraperitoneal injections of ilium xylazil-20 (10 mg. kg^{-1}) and ketamine (50 mg. kg^{-1}) as anaesthesia.

Reduced postnatal growth is primarily caused by poor postnatal nutrition (7, 9). In rats, decreases in litter size also decrease the quality and quantity of milk during lactation and, consequently, slows postnatal growth (49, 56) which leads to catch-up growth and increased risk of heart disease (10). Restricted rats have decreased litter sizes (~5 pups) compared to sham-operated Controls (10-14 pups) (49, 62). Therefore, to create a litter-size control for the Restricted group, and provide a model of delayed postnatal growth (relative to the Control group), half of the sham-operated Control rats had their litters size reduced at birth to 5 pups to create a 'Reduced' group. Each growth cohort consisted of 22-24 male rats.

Exercise training and bodyweight

At 5 weeks of age, rats from each growth cohort (Restricted, Reduced, and Control) were randomly divided into 3 exercise groups. The first of the 3 exercise groups remained sedentary for the entirety of the study (24 weeks) to create the 'Sedentary' group. The second underwent 4 weeks of exercise training from 5-9 weeks of age to create the 'Early exercise' group. The remaining third were exercised from 20-24 weeks of age to create the 'Late exercise' group. Therefore, this further division of the 3 growth cohorts into 3 exercise groups created 9 growth/exercise subgroups. Each of the 3 exercise groups consisted of 22-24 male rats, with 7-8 in each of the 9 subgroups. A maximum of three pups were used per dam and each pup was assigned to a different exercise protocol.

Both Early and Late exercise training involved treadmill running 5 days a week. Exercise duration progressively increased from 20 minutes per day at a speed of 15 metres per minute up to 60 minutes for the first week. The following three weeks were all conducted at 20 metres per minute for 60 minutes per day (34, 35, 60). Individual body weight of each animal was determined at day 6 (when the animals were sexed), as well as weeks 2, 5, 9, 16, 20, and 24.

Heart collection and analyses

At 24 weeks of age all animals were euthanized with an intraperitoneal injection of ilium xylazil-20 (30 mg/kg) and ketamine (225 mg/kg). This occurred 72 hours after the final exercise training session in the Late exercise cohort. The entire heart was excised, weighed, and snap-frozen before being crushed into a powder as previously described (60) and stored at -80°C until the extractions. To measure heart weight relative to body weight, the cardiac weight index (CWI) was calculated (body weight (g) / heart weight (mg)). This body weight and heart weight data are subsets of those we have previously published (35, 60).

Genomic DNA was extracted from whole heart tissue using the Purelink Genomic DNA Mini Kit (Life Technologies). Telomere length was measured by quantitative polymerase chain reaction (qPCR) using the telomere (T) to single-copy gene (S) T/S ratio method (15). Briefly, the square of the cycle threshold (Ct) for the single-copy gene ('S') 36B4, which is present only once in the genome, is divided by the squared Ct for Tel1 ('T'), which reflects telomere length. This yields the T/S ratio, a measure of average telomere length.

RNA was isolated with TRI reagent (Life Technologies) and then reverse transcribed into cDNA using the High Transcriptase cDNA kit (Life Technologies). mRNA levels of telomerase reverse transcriptase (Tert), the rate-limiting step of the telomere-lengthening enzyme telomerase (37), and telomerase RNA component (Terc) (48), were assessed. Telomerase activity was not directly measured as other studies have shown that *Tert* mRNA expression strongly correlates with telomerase activity in the rat heart (39, 50).

A key telomeric gene shown to be essential in maintaining telomere length and cell viability in the heart (51) is telomere repeat-binding factor 2 (Trf2). Protein phosphatase 1 regulatory subunit 10 (Ppp1r10, also known, and hereafter referred to, as Pnuts) can facilitate the binding of Trf2 and is protective of cardiac telomere length and apoptosis (13) but its involvement in exercise-induced hypertrophy is not known. Moreover, the histone deacetylase Sirtuin 1 (Sirt1) is also associated with heart disease and aberrations in telomeric proteins (2, 40). As such, the mRNA levels of *Trf2*, *Pnuts*, and *Sirt1* were also assessed.

For all telomere length and gene expression analyses, the SensiFast SYBR Low-ROX Kit (Bioline) was used in a Viia7 qPCR machine (Life Technologies). For gene expression studies, glyceraldehyde-3-phosphate dehydrogenase (Gapdh) was the housekeeping gene as used in Chapter 2 (44). The primers used were also the same as Chapter 2 (44) except for

Trf2 (forward: CTCCACGACAGCCTCGGAA [200nM] and reverse: GGGATGCTAGG TTAGGAAGTACCA [200nM]) and Sirt1 (forward: TGTTTCCTGTGGGATACCTGA [200nM] and reverse: TGAAGAATGGTCTTGGGTCTTT [200nM]) which were designed to flank exon-exon junctions and melt at 60°C using the Primer Blast tool from NCBI.

qPCR conditions for telomere measurement involved one step of 95°C for 10 minutes followed by 36 cycles of 95°C for 15 seconds, 54°C for 20 seconds and 72°C for 2 minutes. For gene expression analyses, samples were heated to 95°C for ten minutes and then underwent 40 cycles of 95°C for 5 seconds, 58°C for 10 seconds, and 72°C for 10 seconds. Following each run protocol, specificity of product amplification was determined by melting curve analysis and DNA gel electrophoresis (data not shown).

Statistical analyses

Two-way analysis of variance (ANOVA) was used to determine the effects of growth and exercise as well as a possible growth/exercise interaction for all variables. Significant differences within growth cohorts and exercise groups were investigated using Bonferroni's correction. Significant interactions were inspected using one-way ANOVAs. Normality and homogeneity of variances were assessed using the Shapiro-Wilk and Lavene tests, respectively. Pearson's (normally distributed) or Spearman's (nonparametric) tests were used to detect significant correlations. Stepwise multiple regression was performed to identify significant predictors of CWI and cardiac telomere length (F-entry probability, 0.05; removal, 0.1). Data were analysed using SPSS version 24 with $P<0.05$ regarded as significant.

Results

Body weight

Restricted rats were significantly lighter than Control and Reduced rats at postnatal day 6 and the beginning of Early exercise training at 5 weeks of age (all $P<0.05$) (Table 1). Reduced rats, however, were not lighter than Controls at any age measured (all $P>0.05$) but had accelerated growth such that they were heavier than Controls from 16 weeks of age onward (all $P<0.05$) (35). At 24 weeks of age, body weight was significantly affected by growth restriction ($P=0.004$) and exercise treatment ($P=0.042$) but there was no interaction effect (Figure 1A). Reduced rats were significantly heavier than Control ($P=0.010$) and Restricted ($P=0.013$) rats (Figure 1A). Early exercise rats were significantly heavier than Late exercise rats ($P=0.037$) with Control rats being intermediate.

Heart weight and cardiac weight index

There was a significant main effect for growth restriction ($P=0.017$) and exercise treatment ($P=0.002$) on heart weight at 24 weeks of age (Figure 1B). There was a trend for Reduced rats to have heavier hearts than Control ($P=0.062$) and Restricted ($P=0.050$) rats. Early ($P=0.001$) and Late ($P=0.046$) exercise significantly increased heart weight relative to the Sedentary group (Figure 1B). Only exercise had a significant overall effect on CWI ($P<0.001$) with no significant interaction or influence of growth restriction detected (Figure 1C). Both Early ($P=0.002$) and Late ($P=0.005$) exercise significantly increased CWI relative to the Sedentary group.

Table 1. Body weight by growth cohort before Early exercise treatment.

Growth cohort	**BW 6 days of age**	**BW 2 weeks of age**	**BW 5 weeks of age**
Control	8.78 ± 0.18	21.70 ± 0.35	80.48 ± 1.13
Restricted	7.68 ± 0.29*$^{\#}$	20.03 ± 0.76$^{\#}$	74.38 ± 2.08*$^{\#}$
Reduced	8.86 ± 0.15	23.27 ± 0.39	80.34 ± 1.27

Data are presented as *M* ± *SEM*, *n*=22-24 per growth cohort. BW, body weight. **P*=<0.05 compared to Control, $^{\#}P$<0.05 compared to Reduced, both Bonferroni comparisons of main effects.

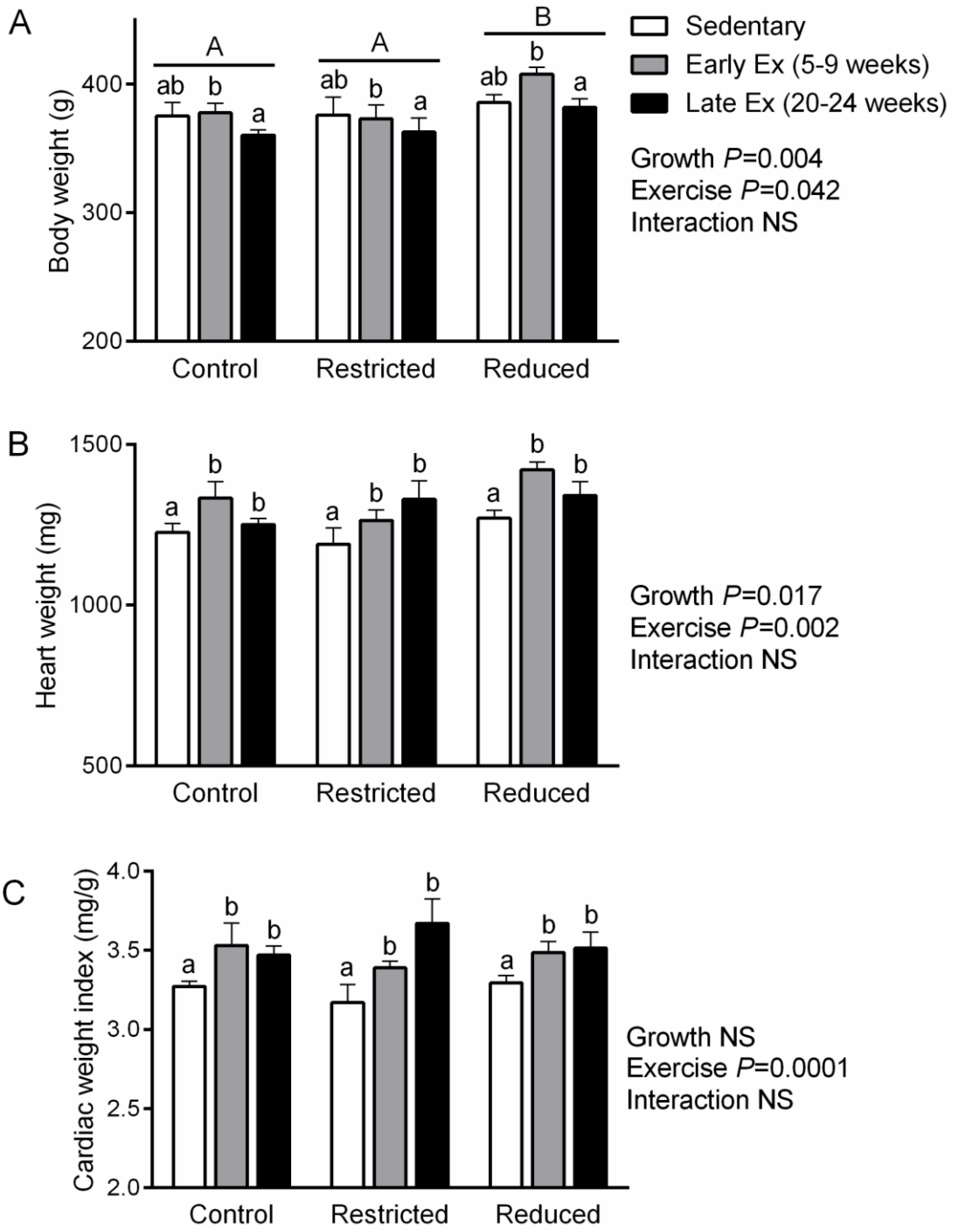

Figure 1. Heart weight, body weight, and cardiac weight index. (A) Reduced growth and Early exercise increased body weight; (B) Early exercise increased heart weight; and (C) Early and Late exercise increased CWI. All at 24 weeks of age. Clear bars indicate Sedentary, lightly shaded bars indicate early exercise (Early Ex), and dark bars indicate late exercise (Late Ex) groups. Data are presented as mean ± *SEM* (*n* = 7-8/group). Significant differences for Growth Restriction effects are denoted by capital letters where 'B' is significantly higher than 'A'. Lower case letters refer to Exercise effects where 'b' is significantly higher than 'a' with 'ab' intermediate and not significantly different from 'a' or 'b'. All Bonferroni comparisons of main effects; $P<0.05$. NS, not significant. This data is a subset of what has been previously published (60).

Telomere length and telomerase genes

Both growth restriction (P<0.001) and exercise treatment (P<0.001) significantly influenced cardiac telomere length (T/S ratio) (P<0.001, main effect of exercise) (Figure 2A). Restricted rats had significantly shorter cardiac telomeres than Control and Reduced rats (Figure 2A). Rats treated with Early exercise had significantly longer cardiac telomeres than Sedentary (P<0.001) and Late (P=0.001) exercise groups (Figure 2A). Cardiac telomere length was significantly correlated with bodyweight (r=0.371, P=0.001) and heart weight (r=0.289, P=0.012) but not CWI (r=0.010, P=0.931).

Growth restriction (P=0.022), but not exercise or the interaction between the two, had a significant effect on *Tert* mRNA levels (Figure 2B). *Tert* expression was significantly higher in Reduced rats compared to Controls (P<0.001) with Restricted rats being intermediate (Figure 2B). Interestingly, *Tert* expression was not correlated with telomere length (ρ=-0.011, P=0.931).

There was no significant effect of growth restriction, exercise treatment, or interaction between the two on *Terc* mRNA levels (Figure 2C). Cardiac *Tert* and *Terc* mRNA levels were positively correlated (ρ=0.414, P<0.001) however *Terc* expression was not correlated with telomere length (ρ=-0.102, P=0.410).

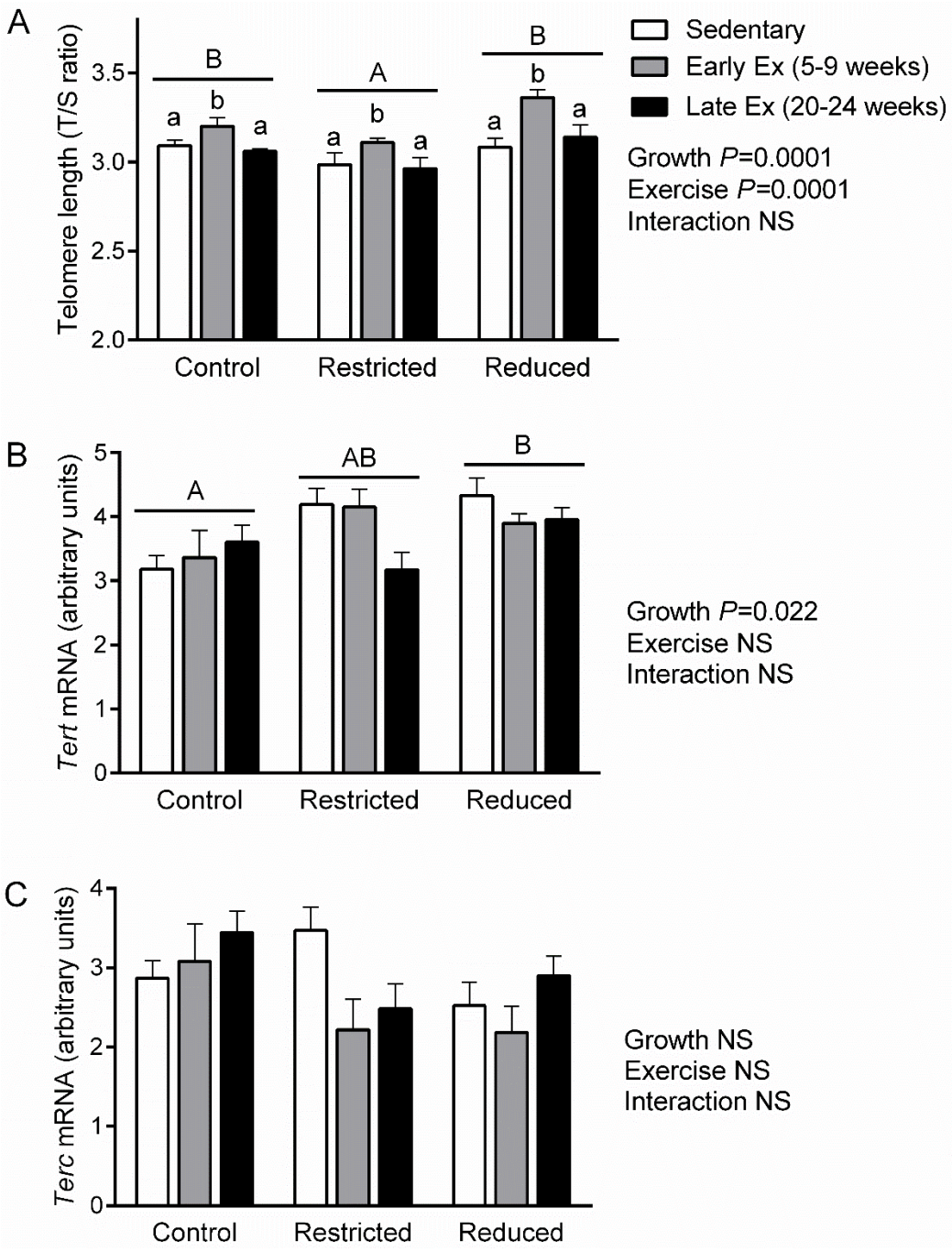

Figure 2. Telomere length and telomerase genes. (A) Telomere length (T/S ratio) was shorter in Restricted rats but longer following Early exercise; (B) *Tert* expression was higher in Reduced rats; and (C) *Terc* mRNA levels did not change because of Growth restriction or Exercise treatment. All whole-heart tissue at 24 weeks of age. Clear bars indicate Sedentary, lightly shaded bars indicate early exercise (Early Ex), and dark bars indicate late exercise (Late Ex) groups. Data are presented as mean ± *SEM* (*n* = 7-8/group). Significant differences for Growth Restriction effects are denoted by capital letters where 'B' is significantly higher than 'A' with 'AB' intermediate and not significantly different from 'A' or 'B'. Lower case letters refer to Exercise effects where 'b' is significantly higher than 'a'. All Bonferroni comparisons of main effects; $P<0.05$. NS, not significant.

Telomere-related genes

Trf2 expression was not affected by growth restriction or exercise treatment but the interaction between the two was significant (P=0.032) (Figure 3A). There was a significant effect of growth restriction within the Sedentary group (P=0.011) with Restricted and Reduced rats having higher *Trf2* expression than Controls (P<0.05) (Figure 3A). Exercise also had a significant effect of on cardiac *Trf2* expression within the Control cohort (P=0.044) but post hoc analysis did not reveal significant differences between the exercise groups.

There was a significant effect of growth restriction (P=0.042) but not exercise treatment, on *Pnuts* mRNA levels (Figure 3B). Control rats had significantly lower cardiac *Pnuts* mRNA levels than Restricted rats with Reduced rats intermediate. *Pnuts* and *Trf2* expression were strongly correlated (r=0.589, P<0.001).

There was a significant effect of growth restriction (P=0.021) and growth restriction/exercise treatment interaction (P=0.001) but not exercise treatment on *Sirt1* mRNA levels (Figure 3C). In Sedentary rats, there was a significant effect of exercise (P<0.001) with Restricted and Reduced having higher *Sirt1* expressions than Controls (P<0.05) (Figure 3C). There were significant effects of exercise for Control rats (P=0.0001) with Late exercise having higher *Sirt1* mRNA than Sedentary and Early exercise intermediate (P<0.05); and Reduced rats (P=0.008), with Early and Late exercise having lower *Sirt1* mRNA than Sedentary (P<0.05) (Figure 3C). *Sirt1* expression was also positively correlated with *Trf2* (r=0.642, P<0.001) and *Pnuts* (r=0.709, P<0.001) expression.

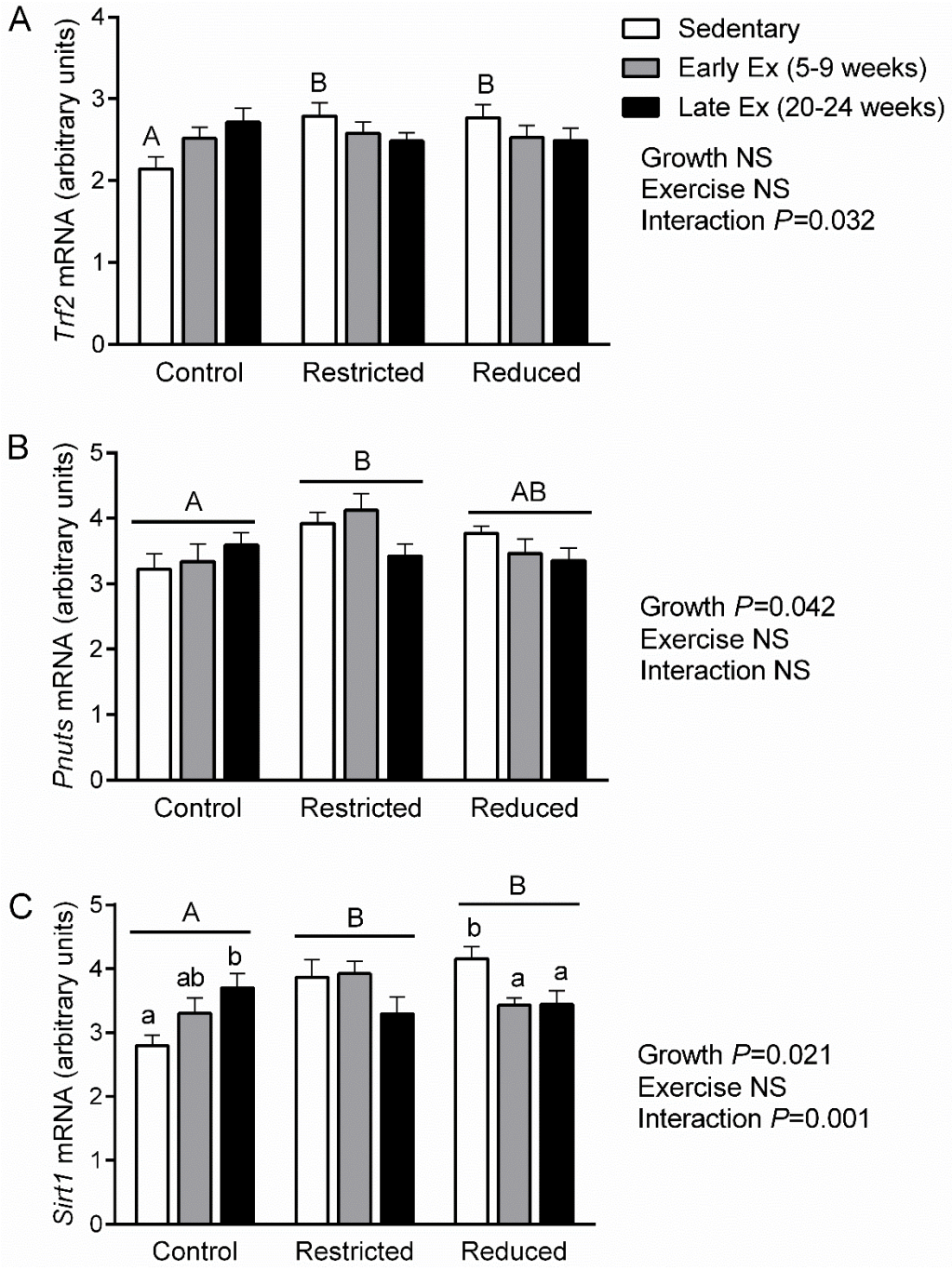

Figure 3. Telomere-related genes. (A) *Trf2* expression was higher in Restricted and Reduced Sedentary rats relative to Control Sedentary rats; (B) *Pnuts* expression was higher in Restricted rats; and (C) *Sirt1* mRNA levels were higher in Restricted and Reduced rats compared to the Controls. All whole-heart tissue at 24 weeks of age. Clear bars indicate Sedentary, lightly shaded bars indicate early exercise (Early Ex), and dark bars indicate late exercise (Late Ex) groups. Data are presented as mean ± *SEM* (*n* = 7-8/group). Significant differences for Growth Restriction effects are denoted by capital letters where 'B' is significantly higher than 'A' with 'AB' intermediate and not significantly different from 'A' or 'B'. Lower case letters refer to Exercise effects where 'b' is significantly higher than 'a' with 'ab' intermediate and not significantly different from 'a' or 'b'. All Bonferroni comparisons of main effects; $P<0.05$. NS, not significant.

Predictors of cardiac weight index and cardiac telomere length

Stepwise multiple regression analysis revealed that Early and Late exercise were the only significant predictors of CWI. Both exercise regimes had a positive effect on relative heart weight, accounting for 18.5% of the variation in CWI (adjusted R^2=0.185) (P=0.001) (Table 2). For cardiac telomere length, Early exercise (positive effect) and Restricted growth (negative effect) were the only significant predictors and together explained 36.9% of the variation in T/S ratio (adjusted R^2=0.369) (P<0.001) (Table 3).

Table 2. Significant predictors of cardiac weight index.

CWI	β ± S.E.	Standardised β	95% C.I. for β	*P* value
Late exercise	0.305 ± 0.075	0.526	0.156 to 0.455	<0.001
Early exercise	0.181 ± 0.072	0.323	0.037 to 0.326	0.015

C.I., confidence interval, S.E, standard error of the mean. Covariates included cardiac *Tert*, *Terc*, *Pnuts*, *Trf2*, and *Sirt1* mRNA levels, cardiac telomere length, Restricted (compared to Control), Reduced (compared to Control), Early exercise (compared to Sedentary), and Late exercise (compared to Sedentary). F-entry probability: 0.05, removal: 0.1.

Table 3. Significant predictors of cardiac telomere length.

T/S ratio	β ± S.E.	Standardised β	95% C.I. for β	*P* value
Early exercise	0.171 ± 0.035	0.486	0.102 to 0.240	<0.001
Restricted	-0.136 ± 0.035	-0.382	-0.206 to -0.066	<0.001

C.I., confidence interval; S.E., standard error of the mean. Covariates included cardiac *Tert*, *Terc*, *Pnuts*, *Trf2*, and *Sirt1* mRNA levels, CWI, Restricted (compared to Control), Reduced (compared to Control), Early exercise (compared to Sedentary), and Late exercise (compared to Sedentary). F-entry probability: 0.05, removal: 0.1.

Discussion

The present study sought to identify the effects of growth restriction and exercise treatment on cardiac mass and telomeres in male rats. Growth restriction shortened cardiac telomere length and Early exercise increased heart weight and prevented cardiac telomere attrition relative to Sedentary and Late exercise groups. Importantly, increased cardiac telomere length detected 15 weeks after early exercise occurred in all three growth cohorts, suggesting that early intervention with exercise treatment can increase cardiac telomere length in both normal and growth-restricted individuals. This finding also provides further evidence that this early exercise regime induces a physiological and not pathological increase in heart mass in adulthood. Indeed, a recent study using the same early exercise protocols on male rats found that early exercise increases cardiomyocyte proliferation and cardiac mass without affecting cardiac function, chamber size, or cardiac fibrosis levels (5). These findings highlight the utility of early exercise to improve cardiac health in later life.

An increase in cardiac telomere length has important consequences in terms of cardiovascular health as only cardiomyocyte precursor cells with long telomeres can produce functional and long-living daughter cells (30). Indeed, one explanation for the increase in telomere length following early exercise is an increase in the number of young cardiomyocytes, as we have recently reported in 24-week-old WKY rats treated with Early exercise (5). The increased complement of cardiomyocytes in the adult heart following this training regime supports the concept that these early-life exercise interventions increased the number of proliferating cardiomyocytes with long telomeres within the myocardium (38, 63). This could also explain why the Late exercise groups did not have longer telomeres. In accordance with this finding, there was no difference in the number of cardiomyocytes as a result of Late exercise (5), suggesting that the increase in cardiac mass and relative heart

weight in the Late exercise groups in the present study is due to cardiomyocyte hypertrophy and not hyperplasia. Similarly, cardiomyocyte number is critical in the regards to cardiac health, with a cardiomyocyte apoptotic rate of only 0.023% considered pathologically significant (64). This suggests that even a small decrease in average cardiac telomere length may significantly affect cardiomyocyte viability and thus cardiac function. Future studies that directly measure cardiomyocyte size and proliferation are required to confirm the effects of exercise on the heart at different ages.

Another key finding of this study was that Restricted rats had shorter cardiac telomeres than Reduced and Control rats in all exercise groups. Interestingly, in the hypertrophic heart rat studied in Chapter 2, body weight was also lighter throughout life and cardiac telomere length shorter at 13 weeks of age (44). This suggests that both prenatal and postnatal growth restriction are needed to shorten cardiac telomere length and that low birth weight compounds the risk of cardiovascular disease. Importantly, these findings are not confounded by typical risk factors for cardiovascular disease and short telomeres, such as obesity (46) and old age (32). In contrast to the findings of Chapter 2 (44), however, CWI was not correlated with the T/S ratio in the present study so a relationship between cardiac telomere length and relative heart weight could not be determined. Indeed, only exercise treatment and Restricted (but not Reduced) growth were identified as significant predictors of CWI and telomere length in the present study.

The similar telomere lengths detected in the Sedentary and Late exercise groups is consistent with previous research showing that changes in telomere length take time to occur. For instance, there were no differences in cardiac telomere length between heart/muscle-specific manganese superoxide dismutase-deficient mice (H/M-SOD2$^{-/-}$), a model of premature heart failure, and the control strain after 4 weeks of antioxidant treatment (41). In contrast, 8 weeks of antioxidant treatment rescued the decreased telomere length observed in

untreated H/M-SOD2$^{-/-}$ mice (52). Similarly, telomere length in the heart was unchanged in mice immediately following 3 weeks of exercise (65) whereas 44 weeks of exercise resulted in significantly longer cardiac telomeres in rats (39). The current study demonstrates that a short-term exercise treatment in early life can significantly increase cardiac telomere length. Indeed, this early exercise training protocol is analogous to early adolescence in humans which suggests that exercise interventions in school curricula could help prevent heart disease even if exercise is not continued into adulthood (5). Benefits may also be possible in later life, but we could not determine this, as the present study concluded only three days after Late exercise treatment ended. Longer studies could confirm if exercise interventions in adulthood produce lasting effects into later life.

In contrast to telomere length, changes in Tert and Trf2 can occur after only short treatments. Indeed, telomerase activity and Trf2 protein levels are downregulated in H/M-SOD2$^{-/-}$ mice but this can be inhibited with only 4 weeks of antioxidant treatment (before changes in telomere length are observed) (41). In the present study, *Tert* expression was higher in Reduced compared to Control rats and *Trf2* expression increased in Restricted and Reduced Sedentary rats relative to Control Sedentary rats. Similarly, *Pnuts* expression was higher in Restricted and *Sirt1* higher in Restricted and Reduced rats compared to the Controls. These changes can all be viewed as compensatory responses to the stresses of Restricted and Reduced growth or the benefits of exercise. In support of this suggestion, there is much evidence that *Tert* expression increases at the onset of cardiac dysfunction but returns to basal levels or lower after chronic stress (reviewed in (14)). Similarly, Tert protein levels increase after three weeks of voluntary running in mice (65) but no differences were found in rats after 44 weeks of exercise (39). In contrast, *Terc* is often relatively unchanged in animal models of heart disease (17, 41, 44), as it was in the present study. Despite these trends, there

is still little evidence to suggest that these telomeric genes have the sensitivity and/or stable expression to identify cardiac hypertrophy while it is still reversible.

Due to the low mitotic index in the heart, oxidative stress has been suggested to account for the majority of cardiac telomere attrition throughout life (29, 36). Indeed, this is a leading hypothesis regarding the utility of antioxidants to preserve both telomere length (58) and heart function (1). Interestingly, improved anti-oxidant capacity is also an adaptive response to exercise (65) and thus decreased telomere oxidation could explain the longer telomere length found after chronic exercise training. In this regard, our previous research has shown that Reduced and Restricted rats have dysregulated antioxidant as well as metabolic genes in the heart during postnatal life, although these normalise by five weeks of age (with the exception of the antioxidant enzyme manganese superoxide dismutase in males) (61). As telomere length is negatively associated with metabolic dysregulation (59) and oxidative stress (58), this could pre-dispose individuals with perinatal growth restriction to accelerated telomere attrition, leading to premature cell loss and organ failure. In this regard, the opposing effects of exercise (39, 65) could rescue cells from oxidative and metabolic injury, thereby preventing cardiac decline in this model (Figure 4).

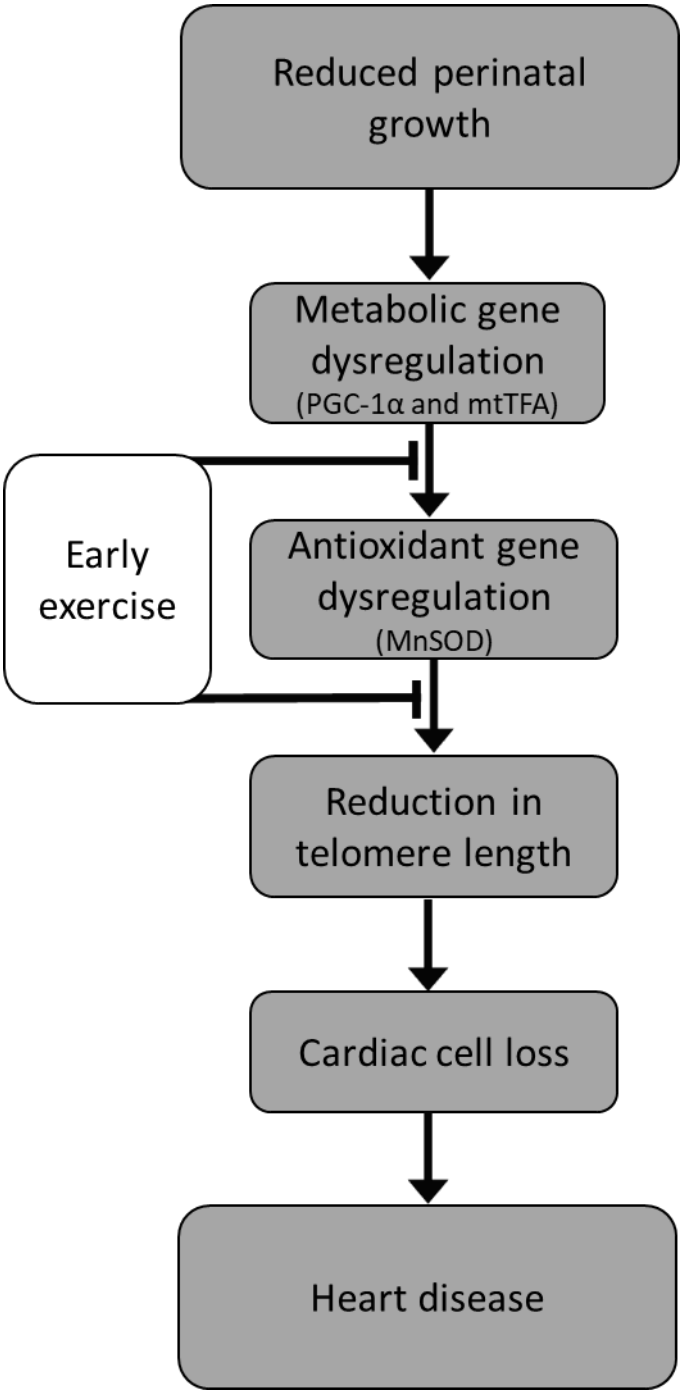

Figure 4. A simplified pathway of the proposed development of heart disease in Restricted male rats and the possible protective effects of early exercise. PGC-1α, peroxisome proliferator-activated receptor gamma coactivator 1-alpha; mtTFA, mitochondrial transcription factor A; mnSOD, manganese superoxide dismutase. This Figure is based on data from the present study and Wadley, et al. (61).

As there are many risk factors for short telomere length and heart disease (26), there are likely several pathways that collectively contribute to cardiac dysfunction, with some being specific to certain types of heart disease or present only at specific stages or in some species. Unlike the dynamic expression of telomeric genes, variables such as increased heart weight (32) and decreased telomere length (31), seem to be universal markers of poor cardiac function despite taking longer to present. In this regard, there is a need for future research to ascertain the survivability of Restricted and Reduced rats compared to Controls and determine if exercise treatment does increase lifespan and delay the onset of cardiovascular disease. Furthermore, detailed analyses of cardiac phenotype and function, such as measurements of cardiac output and histological assessments, of exercised and sedentary rats are required to confirm the relationship between telomere length and the type of cardiac hypertrophy being studied. It would also be interesting to determine telomere length and the levels of telomeric genes at different stages of development and in other tissues. Indeed, accelerated telomere attrition in other tissues may be involved in pathologies, such as diabetes (34) and kidney disease (66), which are also associated with fetal growth restriction.

In regards to cardiac telomere length, much research has focused specifically on cardiomyocytes (16, 36, 51, 55, 58), the functional cells the produce the contractions. The present study, however, along with others (29, 39, 67), used whole-heart tissue which contains endothelial cells, fibroblasts, and leukocytes (47, 53). Telomere length in separated cardiomyocytes, however, was shown to strongly correlate with telomere length in whole heart tissue in Chapter 2 (44). Nevertheless, there is a strong intra-individual variation in telomere length (21) to the extent that there are significant differences in telomere length between cardiomyocytes from the same individual (55). Therefore, the use of whole heart tissue has some limitations but provides telomere measurements at the site of interest, instead of simply using circulating leukocytes (the most common approach (3)) because they can be

easily acquired. Importantly, the present study reveals that Early exercise increases cardiac telomere length in Control, Restricted, and Reduced rats, likely protecting the heart and attenuating the adverse cardiac effects induced by prenatal and postnatal growth restriction. Given the reliability of this finding, we would predict that the effect of Early exercise would be more pronounced if telomere length was only measured in cardiomyocytes.

In conclusion, the present study demonstrates that low birth weight after fetal growth restriction reduces cardiac telomere length. Importantly this can be attenuated by exercise early in life, however, the molecular mechanisms regulating cardiac telomere length remain mostly elusive as there were no reliable correlations with telomeric genes at 24 weeks of age. Nonetheless, such findings suggest there are key pathways that elicit changes in heart weight and cardiac telomere length in response to exercise which could be targeted for therapeutic benefit. Although future studies are required to determine the effects of exercise on the heart at different ages and into later adulthood, our findings, combined with the well-established degenerative nature of heart disease, highlight the importance of early interventions for susceptible individuals. Indeed, exercise may only be a viable treatment option before telomeres reach a critical length and irreversible cell loss ensues.

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Chapter 4

Human Cardiomyocyte Telomeres after Inducing Hypertrophy

Abstract

Background and aims: Cardiomyocyte hypertrophy is a hallmark cellular defect of heart disease. Association studies have shown that cardiomyocyte size correlates with decreases in telomere length and maintenance, but possible causal relationships remain unknown. The aim of the present study was to determine whether changes in cardiomyocyte size effect telomeres and the mechanism/s through which this might occur.

Methods and results: Hypertrophy was induced in human primary cardiomyocytes (HPCs) using the hypertrophic agonists adrenaline, angiotensin II, endothelin-1, isoproterenol, and phenylephrine. Cell size, proliferation, and oxidative stress were determined using immunocytochemistry and/or confocal microscopy. Telomere length and the expression of telomeric, hypertrophic, and inflammatory genes were assessed with qPCR. All treatments significantly increased HPC size ($P<0.05$) but only Endothelin-1 had a significant effect on telomeres, which were shorter after 48 hours, 10 days, and 16 days of treatment ($P<0.05$). ET-1 also induced oxidative stress ($P=0.004$) while all agonists caused increased inflammation, proliferation, and dysregulated metabolic gene expression ($P<0.05$).

Discussion and conclusions: The present study demonstrates that cardiomyocyte hypertrophy can occur independently of changes in telomere length and maintenance. The increase in inflammation and proliferation provide an explanation for the telomere attrition observed in late-stage cardiac hypertrophy and heart failure. Endothelin-1, however, may play a more direct role in cardiomyocyte aging and viability which could be targeted for therapeutic use.

Introduction

All organs of the body rely on the constant circulation of blood which has made cardiomyocytes, the contractile cells of the heart, extremely responsive to stimuli that communicate the needs of other tissues (76). Although most physiological demands can be met by a temporary rise in heart rate, extreme or sustained stimulation to boost cardiac output causes an increase in cardiomyocyte size (21). As such, a certain degree of cardiomyocyte growth is involved in normal development (maturational hypertrophy) as well as strenuous exercise and pregnancy (physiological cardiac hypertrophy) (44). In contrast, pathological stress on the cardiovascular system, such as hypertension (13), atherosclerosis (52), and obesity (90), leads to overt cardiomyocyte growth which causes an abnormal increase in heart mass known as cardiac (or left ventricular) hypertrophy. Therefore, cardiomyocyte hypertrophy is a hallmark cellular defect of heart (or cardiovascular) disease, the worldwide leading cause of death (50).

Excessive stress on the cardiovascular system is associated with an increase in the release of hypertrophic agonists, such as angiotensin-II (Ang-II) (88) and endothelin-1 (ET-1) (3). This activates adrenergic receptors (ARs) on cardiomyocytes (25), leading to alterations in gene expression reminiscent of the fetal and neonatal period (64). Indeed, the most important markers of cardiac hypertrophy and subsequent heart failure are members of the so-called fetal gene program, such as the peptide hormones atrial and brain natriuretic peptide (ANP and BNP) (20). In contrast, physiological hypertrophy boosts cardiac output and is considered harmless (48), typically presenting with the upregulation of mitogen-activated protein kinase 1 (MAPK1) (the gene for ERK2) (9), mechanistic target of rapamycin (MTOR) (68), and protein kinase AMP-activated catalytic subunit alpha 2 (PRKKA2) (91). Despite these important distinctions, all types of cardiac hypertrophy result from stimuli

inducing transcriptional changes that increase intra-cellular protein levels (particularly the key contractile unit α-sarcomeric actin) and thus cardiomyocyte size.

Cardiomyocyte hypertrophy is also accompanied by cell behaviours associated with senescence and apoptosis, such as morphological changes in cytoskeletal (60) and nuclear (57) structure. In this regard, both senescence and apoptosis are controlled by telomere length (2, 5), the number of TTAGGG repeats that protect chromosome ends (6, 19). Indeed, several studies have found that short telomeres (i.e. fewer protective repeats) are associated with cardiomyocyte hypertrophy (12, 45, 74). In addition, the upregulation of the telomere-lengthening enzyme telomerase accompanies the onset of heart disease (75) whereas a reduction is found in its later stages (36, 70). Moreover, reduced expression of proteins that bind to the telomeric sequence, such as telomere repeat-binding factor 2 (TRF2), is observed in diseased hearts (59). In contrast, exercise preserves cardiac telomere length (42), cardiomyocyte viability (58), as well as telomerase and TRF2 expression levels (81). Taken together, these findings suggest that telomeric factors may play an important role in the development of pathological but not physiological cardiac hypertrophy.

Despite several studies, it is still not known whether telomere attrition is a cause, consequence, or epiphenomenon of cardiomyocyte hypertrophy and there are several reasons for this. Firstly, systemic treatments that induce cardiac hypertrophy *in vivo* also induce confounding diseases such as hypertension (61) and renal failure (82). Secondly, findings from animal studies must be interpreted with caution as there are significant interspecies differences in telomere length (66, 75), nuclear morphology, and cardiac histology (37). Thirdly, oxidative stress (75), inflammation (46), and metabolic aberrations (55), are known to decrease telomere length and heart function, however these confounding factors are often not measured or controlled for (23, 65). Finally, it is not always directly established if the hypertrophy being studied reflects the pathological type and is likely to progress to heart

failure. The aim of the present study is to induce hypertrophy in human primary cardiomyocytes (HPCs) *in vitro* and measure the effect on telomere length and maintenance. Importantly, measurements of key confounding factors and markers of different types of cardiac hypertrophy will also be determined. It is hypothesised that the hypertrophic response will cause alterations in telomeric regulators, oxidative stress, metabolic gene expression, and inflammation before significant changes in telomere length are detected.

Methods

Ethical approval

As this study used a cell line, no ethical clearance was required.

Cell culture

The present study used human primary cardiomyocytes (HPCs) from a healthy 56-year-old Caucasian male grown in ready-to-use myocyte growth medium (both from Promo Cell, Heidelberg, Germany). As cardiomyocyte mitosis is very minimal in adult humans (69), this cell line is derived from adult cardiac progenitor cells and, for research purposes, can be expanded for more than 15 population doublings in culture. Importantly, HPCs are not immortal, as the immortalisation of cell culture lines prevents telomere attrition (7) and thus limits the utility of such models in studies of cellular aging. In this study, passage 4 cells (unless otherwise indicated) were seeded at 25% confluence, and allowed to adhere for 24 hours before treatment. 6-well dishes were used for nucleic acid and protein extraction and single glass-bottom dishes of the same size for imaging (Mattek Corporation, Ashland, USA). All cultures were grown, passaged, and stored using the manufacturer's recommended reagents and protocols.

The common immortalised rat embryonic cardiomyoblast (H9c2) cell line was also used to determine if it could be used to model HPC hypertrophy and telomere dynamics. Passage 7 H9c2 cells were grown in Dulbecco's Modified Eagle's Medium supplemented with 10% (v/v) Fetal Bovine Serum and 1% (v/v) Penicillin/Streptomycin, L-Glutamate (Life Technologies). H9c2 cells were seeded at 15% confluence because of their more rapid population doubling time but were otherwise treated identically to the HPCs.

Induction of hypertrophy

The hypertrophic agonists adrenaline (Adren) (100nM), Ang-II (20µM), ET-1 (10nM), isoproterenol (ISO) (10µM), and phenylephrine (PE) (100µM) (all from Sigma Aldrich) were used to increase cell size. All these agonists have been shown to significantly induce hypertrophy in cardiomyocytes in 48 hours or less (26, 28, 62, 72, 89). Optimisations were performed in HPCs for each agonist using five different concentrations one order of magnitude apart (data not shown). This was carried out due to the vastly different concentrations used in previous studies (63, 88). The concentrations used in this study were those which induced the greatest increase in HPC size. A synergistic treatment was also performed with each agonist at 10% of its solo concentration because additive effects have been reported (87). The lower concentration for this treatment was used to minimise the risk of off-target effects, such as the induction of apoptosis, and to preserve reagents.

Cells were treated at zero and 24 hours, then collected at 48 hours for various downstream applications as described below. If significant differences in telomere length were found, 24-hour treatments continued and telomeres were measured again after 10 and 16 days. For telomere length and gene expression analyses, each treatment was performed in triplicate to provide enough data for statistical analyses.

Cell size and proliferation

Immunocytochemistry

Following the treatments, HPCs were washed twice in phosphate buffered saline (PBS), fixed in 4% paraformaldehyde for 30 minutes, washed four times in PBS, then blocked and permeablised in 0.75% 2mg/mL BSA (Thermo Fischer), 2% horse serum, and 0.4% Tx100 in PBS for 30 minutes all at room temperature. Cells received another two washes in PBS then the anti-α-sarcomeric actin monoclonal antibody (Sigma Aldrich) 1:100 (for hypertrophy) or

anti-Ki67 antibody with conjugated fluorescent tag (Abcam) 1:100 (for proliferation) was applied overnight in blocking and permeablisation buffer (see above) at 4ºC (and away from light until imaging was completed). The next morning, cells containing the α-sarcomeric actin antibody were washed four times in PBS then probed with AP130C Goat Anti-Mouse IgG & IgM Antibody, Cy3 conjugate (Merck Millipore) 1:100 for two hours in blocking and permeabilisation buffer (see above) at room temperature. All cells were washed twice in PBS and then Hoerscht (Life Technologies) (1:500 in PBS) was applied for five minutes (and remained at room temperature until after imaging). Finally, all cells were washed four times in PBS and then imaged in PBS.

Confocal microscopy

A Nikon Eclipse Ti-E confocal microscope was to create tomographic images by capturing a section every 1μm over a 15μm z-range. For cell size measurements, five z-stacks were taken and a minimum of 25 cells were included in each group. To determine proliferation, seven z-stacks were captured, each with a minimum of 10 cells. Images were saved as TIFF (for analysis) and JPG (for presentation) files. For cell size measurements, surface area (defined as α-sarcomeric actin fluorescence) was quantified using image J software. This entailed compression into a single image for integrated density quantification. To assess levels of proliferation, cells expressing the Ki67 antigen were divided by the total number of cells per image.

Live cell imaging for reactive oxygen species

Oxidative stress was measured using the Image-iT LIVE Green Reactive Oxygen Species Detection Kit (Life Technologies) and HPC reagents (Promo Cell) according to the manufacture's protocol. Single images were taken (to avoid photobleaching) on a Nikon

Eclipse Ti-E confocal microscope system with a 37°C chamber. Total fluorescence was determined using image J and a minimum of 25 cells were analysed per group (as above).

Telomere Length

DNA extraction

Cells were washed in PBS then scraped into 200uL of PBS using a rubber policeman and stored at -20ºC. Genomic DNA was later extracted from three independent preparations using the Purelink Genomic DNA Mini Kit (Life Technologies) as per the manufacturer's instructions and diluted to 2.5ng/µL based on quantitation with a Nanodrop 2000. A 10ng template of cDNA was then added to a 384-well quantitative polymerase chain reaction (qPCR) plate (Life Technologies) and stored at -20ºC until telomere measurement.

Telomere to Single-copy gene (T/S ratio)

Telomere length was determined using the telomere (T) to single-copy gene (S) T/S ratio method with qPCR as described previously (10). Briefly, the squared cycle threshold (Ct) for 36B4, a single-copy gene ('S') present only once in the genome, is divided by the square of the Ct for Tel1 ('T'), which reflects telomere length. This provides a measure of average telomere length, known as the T/S ratio.

The human primers used are shown in Table 1 and qPCR conditions were one step of 95°C for 10 minutes followed by 36 cycles of 95°C for 15 seconds and 58°C for one minute. Rat primers are shown in Table 2 and measurement involved one step of 95°C for 10 minutes followed by 36 cycles of 95°C for 15 seconds, 54°C for 20 seconds, and 72°C for two minutes. The SensiFast SYBR Low-ROX Kit (Bioline) was used in a Viia7 qPCR machine (Life Technologies) and specificity of amplification was determined by melting curve analysis.

Gene expression

RNA extraction and cDNA synthesis

Cells were washed in PBS, de-attached and mixed with TRI reagent (Life Technologies) by vigorous pipetting, then stored at -80ºC. RNA was later extracted from three independent preparations, quantified with a Nanodrop 2000, then reverse transcribed into cDNA using the High Transcriptase cDNA kit (Life Technologies), and diluted to 5ng/µL. Both protocols were followed as per the manufacturer's instructions. A 20ng template (4µL) was then added to a 384-well qPCR plate (Life Technologies) and stored at -20ºC until the gene expression analyses with qPCR.

Primer design and running conditions

Primers were designed using the NCBI tool Primer Blast (http://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi) to span exon-exon junctions, produce an amplicon of 75-150 base pairs in length, and have a melting temperature as close to 60°C as possible. The SensiFast SYBR Low-ROX Kit (Bioline) was used in a Viia7 qPCR machine (Life Technologies) with β-actin as the housekeeping gene as used previously (73). Amplification specificity was determined by melting curve analysis and DNA gel electrophoresis (data not shown). qPCR conditions involved heating the samples to 95°C for ten minutes followed by 40 cycles of 95°C for 5 seconds, 58°C or 56°C (see Tables 1 and 2) for ten seconds, and 72°C for ten seconds. Selected genes that were reliably dysregulated in HPCs were then evaluated in H9c2 cells to determine the similarities between these two models, with glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) as the housekeeping gene, as used previously (45). Housekeeping genes were also selected because β-actin was more stably expressed than Gapdh in HPCs and Western blot revealed two isoforms of β-actin in H9c2 extracts (data not shown).

Table 1. Human primer information.

Gene name	**Rationale for inclusion**	**Primer sequence 5' → 3'**	**Concentration**	**Annealing temperature**
Tel 1 forward	Reflects telomere length (10)	GGGTTTGTTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTT	300nM	58°C
Tel 1 reverse		GGCTTGCCTTACCCTTACCCTTACCCTTACCCTTACCCT	300nM	58°C
36B4 forward	Single-copy reference gene for *Tel 1 (10)*	CAGCAAGTGGGAAGGTGTAATCC	300nM	58°C
36B4 reverse		CCCATTCTATCATCAACGGGTACAA	500nM	58°C
TERT forward	Catalytic component of telomerase (40)	CAAGCTGTTTGCGGGGATTC	200nM	56°C
TERT reverse		GGGCATAGCTGAGGAAGGTTT	200nM	56°C
TERC forward	RNA-template component of telomerase (56)	ACCCTAACTGAGAAGGGCGT	200nM	58°C
TERC reverse		GGCCAGCAGCTGACATTTTT	200nM	58°C
TRF1 forward	Binds to and protects telomeric DNA (16)	TGCTCGATTTCCTCTGCCTC	200nM	58°C
TRF1 reverse		TGAATAATAGCCTCTGCGCTGT	200nM	58°C
TRF2 forward	Binds to and protects telomeric DNA (79)	AGAACTTGGCCCATCCTGTT	200nM	58°C
TRF2 reverse		GCCTTTTTGGCCATCGTGAG	200nM	58°C
TINF2 forward	Interacts with TRF1 (56)	CCAGGGGGAAGGAAAGGAATC	200nM	58°C
TINF2 reverse		GGTGGCTTCCACAAGCATCT	200nM	58°C
TERF2IP forward	Bridges TRF2 (56)	AGCGGGGAACCACAGAATAAG	200nM	58°C
TERF2IP reverse		GGTGGCTTCCACAAGCATCT	200nM	58°C
TPP1 forward	Binds to single-stranded	ACACGGTGCAAAAATGGCTC	200nM	58°C

TPP1 reverse	telomeric DNA (19)	TCTGCTTGTCGGATGCTCAG	200nM	58°C
POT1 forward	Binds to single-stranded telomeric DNA (19)	AGCAGAGATGGTAAACCTTGC	200nM	58°C
POT1 reverse		TGTTGCTGGAACCAAAGACAT	200nM	58°C
PNUTS forward	Facilitates the binding of TRF2 to telomeres (34)	CGTCCGGGTATAAAAGACTCCA	200nM	58°C
PNUTS reverse		AAATTCAAACCTCTAAACGAACCCC	200nM	58°C
SIRT1 forward	Promotes telomere elongation and genomic stability (18)	CGGTTCCTACTGCGCGA	200nM	58°C
SIRT1 reverse		CCGAACAGAAGGTTATCTGGCT	200nM	56°C
NPPA forward	Key member of the fetal gene program (14)	TAAAAAGCAAGCTGAGGGCG	200nM	56°C
NPPA reverse		ATCTTCAGTACCGGAAGCTGTTA	200nM	58°C
NPPB forward	Key member of the fetal gene program (14)	CTTTCCTGGGAGGTCGTTCC	200nM	58°C
NPPB reverse		GTTGCGCTGCTCCTGTAAC	200nM	58°C
PKCα forward	Causes pathological hypertrophy in rodents (8, 33)	AGAGGGACGTGAGAGAGCAT	200nM	58°C
PKCα reverse		CCTTTGCCACACACTTTGGG	200nM	58°C
NFKB1 forward	Knockout causes pathological hypertrophy in mice (24)	GCTTAGGAGGGAGAGCCCA	200nM	58°C
NFKB1 reverse		TGAAACATTTGTTCAGGCCTTCC	200nM	58°C
MCP-1 forward	Activated by Ang-II in pathological hypertrophy (47)	GAAAGTCTCTGCCGCCCTT	200nM	58°C
MCP-1 reverse		GCATTGATTGCATCTGGCTGAG	200nM	58°C
MTOR forward	Associated with physiological hypertrophy (68)	GAAGCCGCGCGAACCT	200nM	58°C
MTOR reverse		TGGTTTCCTCATTCCGGCTC	200nM	58°C
MAPK1 forward	Upregulated in physiological hypertrophy (9)	TTTGTCAGGACAAGGGCTCAG	200nM	58°C
MAPK1 reverse		CAGGACCAGGGGTCAAGAAC	200nM	58°C
PRKAA2 forward	Promotes physiological	ACCAGGTGATCAGCACTCCA	200nM	58°C

PRKAA2 reverse	hypertrophy (91)	TCTCTTCAACCCGTCCATGC	200nM	58°C
IL-1β forward	Marker of inflammation in cardiomyocytes (84)	GAGACAAAACAAAGAAGGCTGGA	200nM	58°C
IL-1β reverse		AGATAGGTTCTCTGGTGACAATGTA	200nM	58°C
IL-6 forward	Marker of inflammation (84)	CTCAATATTAGAGTCTCAACCCCCA	200nM	58°C
IL-6 reverse		AAGGCGCTTGTGGAGAAGG	200nM	58°C
COX2 forward	Marker of inflammation in cardiomyocytes (71)	AATCCTTGCTGTTCCCACCC	200nM	58°C
COX2 reverse		TCAAAAATTCCGGTGTTGAGCA	200nM	58°C
Mi67 forward	Marker of proliferation in cardiomyocytes (31)	TGCAGCGAACGCGAC	200nM	58°C
Mi67 reverse		CTGGCTCCTGTTCACGTATT	200nM	58°C
B-actin forward	Housekeeping gene (73)	CCTTCTACAATGAGCTGCGTG	200nM	58°C
B-actin reverse		TCAGAGAAGAGAGTCCTGGGTC	200nM	58°C

Tel1, telomere primers 1; *36B4*, acidic ribosomal phosphoprotein PO; *TERT*, telomerase reverse transcriptase; *TERC*, telomerase RNA component; *TRF1*, telomere repeat-binding factor 1; *TRF2*, telomere repeat-binding factor 2; *TINF2*, TRF1 interacting nuclear factor 2; *TERF2IP*, TRF2-interacting protein ; *TPP1*, tripeptidyl peptidase 1; *POT1*, protection of telomeres 1; *PNUTS*, protein phosphatase 1 regulatory subunit 10; *SIRT1*, sirtuin 1; *NPPA*, gene for atrial natriuretic peptide; *NPPB*, gene for brain natriuretic peptide; *PKCα*, phosphokinase C alpha; *NFKB1*, nuclear factor NF-kappa-B p105 subunit; *MTOR*, mechanistic target of rapamycin; MCP-1, monocyte chemotactic protein 1; *MAPK1*, mitogen-activated protein kinase 1; *PRKAA2*, protein kinase AMP-activated catalytic subunit alpha 2; *IL-1β*, interleukin-1β; *IL-6*, Interleukin 6; *COX2*, cyclooxygenase-2; *Mi67*, gene for Ki67.

Table 2. Rat primer information.

Gene name	Primer sequence 5' → 3'	Concentration	Annealing temperature
Tel 1 forward	GGTTTTTGAGGGTGAGGGTGA GGGTGAGGGTGAGGGT	300nM	56°C
Tel 1 reverse	TCCCGACTATCCCTATCCCTA TCCCTATCCCTATCCCTA	300nM	56°C
36B4 forward	CAGCAACTGGGAAGGTGTAATCC	300nM	56°C
36B4 reverse	CCCATTCTATCATCAACGGGTACAA	500nM	56°C
Tert forward	AACTACGAGCGGACCAAACA	200nM	58°C
Tert reverse	CCCTGTCACATCTGCCTTAAC	200nM	58°C
Terc forward	TGTTATAGCTGTGGGTTCTGTTCTT	200nM	58°C
Terc reverse	CCGCTGCAGGTCTGAACTTT	200nM	58°C
Mapk1 forward	AAATTGGTCAGGACAAGGGCTC	200nM	58°C
Mapk1 reverse	CTCGGAACGGCTCAAAGGAG	200nM	58°C
Il-1β forward	CAGCTTTCGACAGTGAGGAGA	200nM	58°C
Il-1β reverse	TGTCGAGATGCTGCTGTGAG	200nM	58°C
Cox2 forward	TGCTGTTCCAACCCATGTCA	200nM	58°C
Cox2 reverse	TCTTGTCAGAAACTCAGGCGT	200nM	58°C
Mi67 forward	GACACCAGACCATGCCAATG	200nM	58°C
Mi67 reverse	GGGTTCTAACTGGTCTTCCTGG	200nM	58°C
Gapdh forward	GGGGCTCTCTGCTCCTCCCTG	200nM	58°C
Gapdh reverse	ACGGCCAAATCCGTTCACACC	200nM	58°C

Tel1, telomere primers 1; *36B4*, acidic ribosomal phosphoprotein PO; *TERT*, telomerase reverse transcriptase; *TERC*, telomerase RNA component; *TRF2*, telomere repeat-binding factor 2; *PNUTS*, protein phosphatase 1 regulatory subunit 10; *SIRT1*, sirtuin 1; *MAPK1*, mitogen-activated protein kinase 1; *IL-1β*, interleukin-1β; *COX2*, cyclooxygenase-2; *Mi67*, gene for Ki67; *Gapdh*, glyceraldehyde 3-phosphate dehydrogenase.

Protein levels

Protein extraction

Cells were washed in PBS then scraped into 300uL of RIPA buffer (Sigma Aldrich) containing 1% Halt Protease & Phosphatase Inhibitor Cocktail (Thermo Scientific) and stored at -80ºC until protein extraction. Once extracted, total soluble protein was then diluted 1:3 and measured with the bicinchoninic acid (BCA) assay kit (Bio-Rad) against a standard curve of bovine serum albumin (BSA) (Thermo Scientific) with eleven points ranging from 0.0 – 2.0 mg/mL. The absorbance at 570nm was determined using a Multiskan Microplate Photometer (Thermo Scientific). All procedures strictly adhered to the manufacturer's instructions.

Western blot

25µg of cell protein was mixed with 5% β-Mercaptoethanol (Sigma Aldrich) in Laemelli Sample Buffer (Bio-Rad) then denatured at 100°C for 5 minutes. Samples were resolved on a 4–15% Mini-PROTEAN® TGX™ Precast Gel (Bio-Rad) at 80 volts for around 90 minutes at room temperature then electroblotted on to a Nitrocellulose Membrane (Thermo Scientific) at 100 volts for one hour at 4°C. The membrane was blocked for one hour at room temperature using 5% skim milk in TBS-Tween 20 before the primary antibody was applied at 4ºC overnight. Four 15-minute washes in TBS-Tween 20 were performed followed by the application of the appropriate secondary antibody in a 0.5% skim milk in TBS-Tween 20 solution for 1 hour at room temperature. The wash steps were then repeated before detection using enhanced chemiluminescence SuperSignal West Pico Substrate (Thermo Scientific). Images were captured with a UVITEC Alliance digital imaging system (Thermo Scientific). To determine levels of apoptosis, the presence of caspase-3 (Cell Signalling #9665) (1:2500) was assessed then the membrane was stripped for 15 minutes at room temperature using

Restore™ Western Blot Stripping Buffer (Thermo Scientific) and re-blocked and probed for cleaved caspase-3 (Cell Signalling #9664) (1:2500).

Data analyses

Microsoft Excel, SPSS for windows, and PRISM were used to organise and transform data, perform statistical tests, and create the graphs, respectively. For confocal microscopy and gene expression experiments, statistical analyses were performed on the least transformed relative data (the integrated density and delta Ct values, respectively) which was later transformed to a fold change for graphical representation. In this regard, all treatment groups are displayed together for simplicity, with one graph per dependent variable. Each treatment group, however, is compared only to the control using the appropriate *t*-test. All data are presented as means (*M*) ± the standard deviation (*SD*) with $P<0.05$ regarded as significant.

Results

Validation of hypertrophy

In HPCs, all agonists significantly increased cell size ($P<0.05$) compared to the control (Figure 1). Interestingly, a synergistic treatment (each agonist at 10% of its solo concentration) caused a significant decrease in HPC size ($P=0.005$) relative to the control (Figure 1). Similarly, in H9c2 cells, all agonists significantly increased cell size ($P<0.05$) compared to the control (Figure 2).

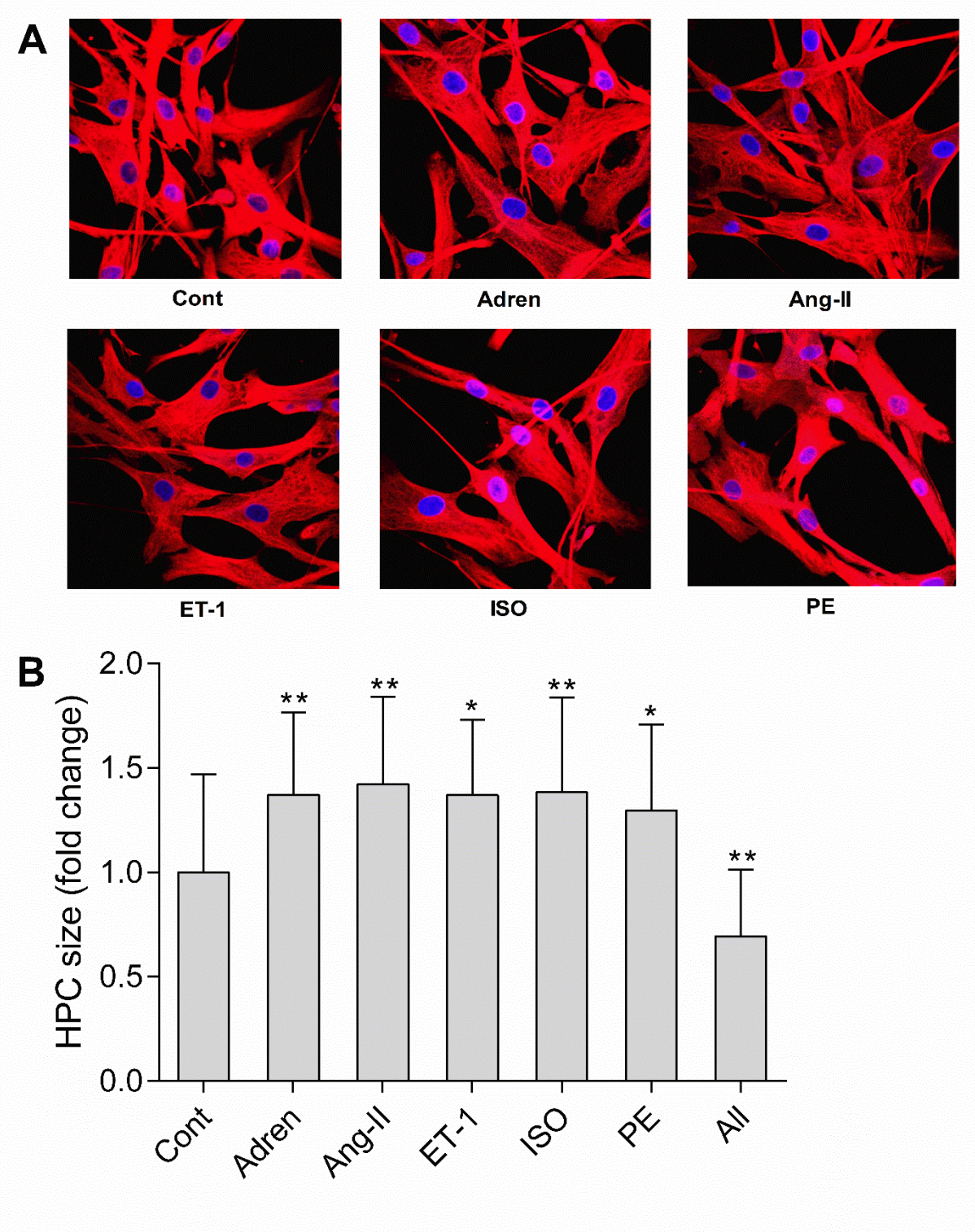

Figure 1. HPC size. (A) Anti-α-sarcomeric actin immunocytochemistry on HPCs; (B) Agonist stimulation increased HPC size. Data are presented as mean ± SD (n=25) with Cont. normalised to 1. * indicates $P<0.05$ and ** $P<0.01$ compared to Cont. Cont, control; Adren, adrenaline; Ang-II, angiotensin-II; ET-1, endothelin-1; ISO, isoproterenol; PE, phenylephrine; All, all agonists.

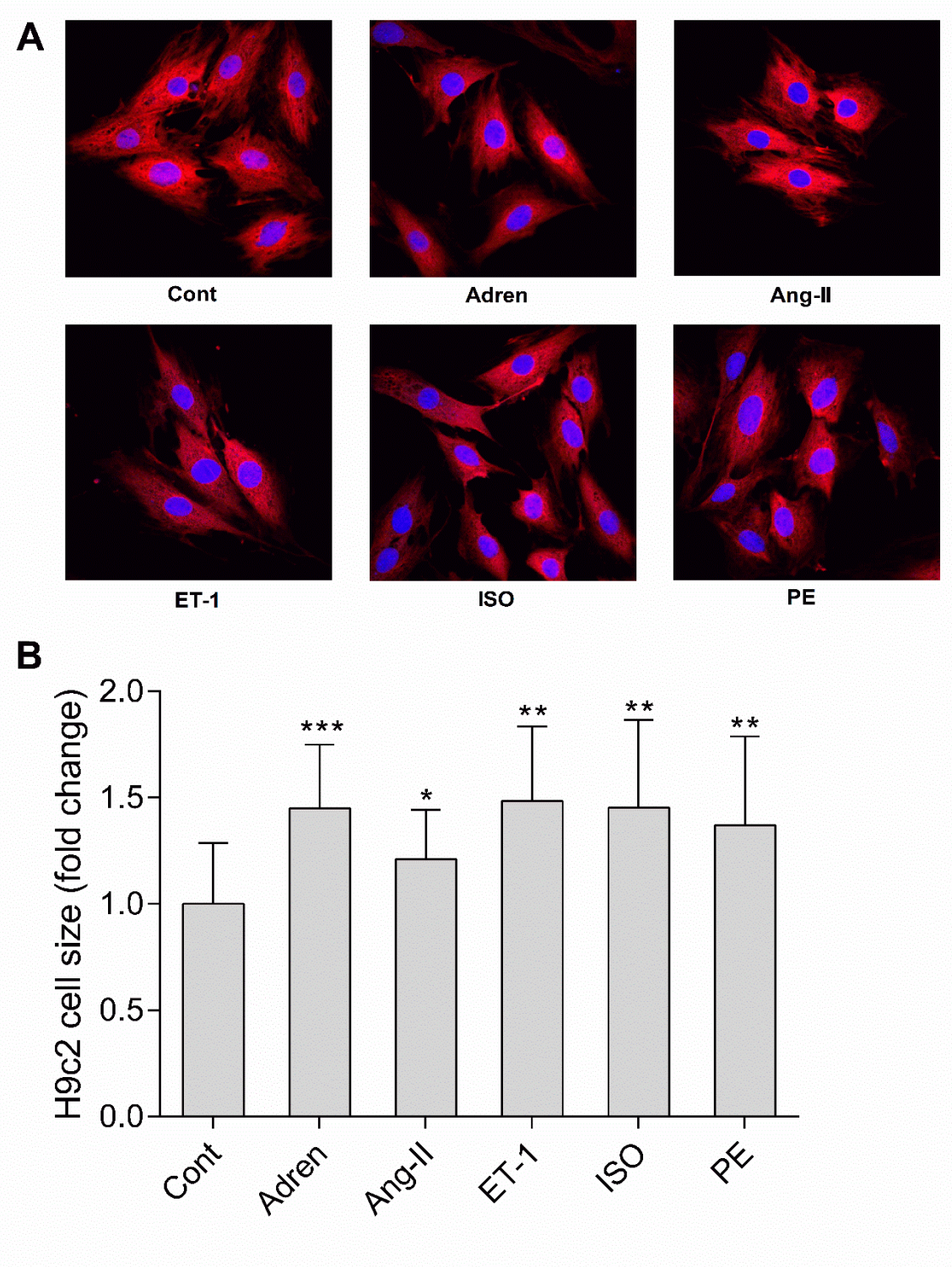

Figure 2. H9c2 cell size. (A) Anti-α-sarcomeric actin immunocytochemistry on H9c2 cells; (B) Agonist stimulation increased H9c2 cell size. Agonist stimulation increased H9c2 cell size Data are presented as mean ± SD (n=25) with Cont. normalised to 1. * indicates $P<0.05$, ** $P<0.01$, and ** $P<0.001$ compared to Cont. Cont, control; Adren, adrenaline; Ang-II, angiotensin-II; ET-1, endothelin-1; ISO, isoproterenol; PE, phenylephrine.

Telomere length

In HPCs, ET-1 caused a significant reduction in HPC telomere length after 48 hours (P=0.010). Telomere length continued to shorten in response to ET-1 treatment for 10 (P=0.034) and 16 (P=0.044) days (Figure 3A). In contrast, there were no significant differences in telomere length between adrenaline, angiotensin-II, isoproterenol, phenylephrine, and the control cells after 48-hours of treatment (Figure 3B). Similarly, the synergistic treatment did not alter telomere length and as this also failed to increase cell size, no further experiments were carried out on this group. In H9c2 cells, there were no significant differences in telomere length between the control cells and any of the groups after 48 hours of treatment (Figure 4A).

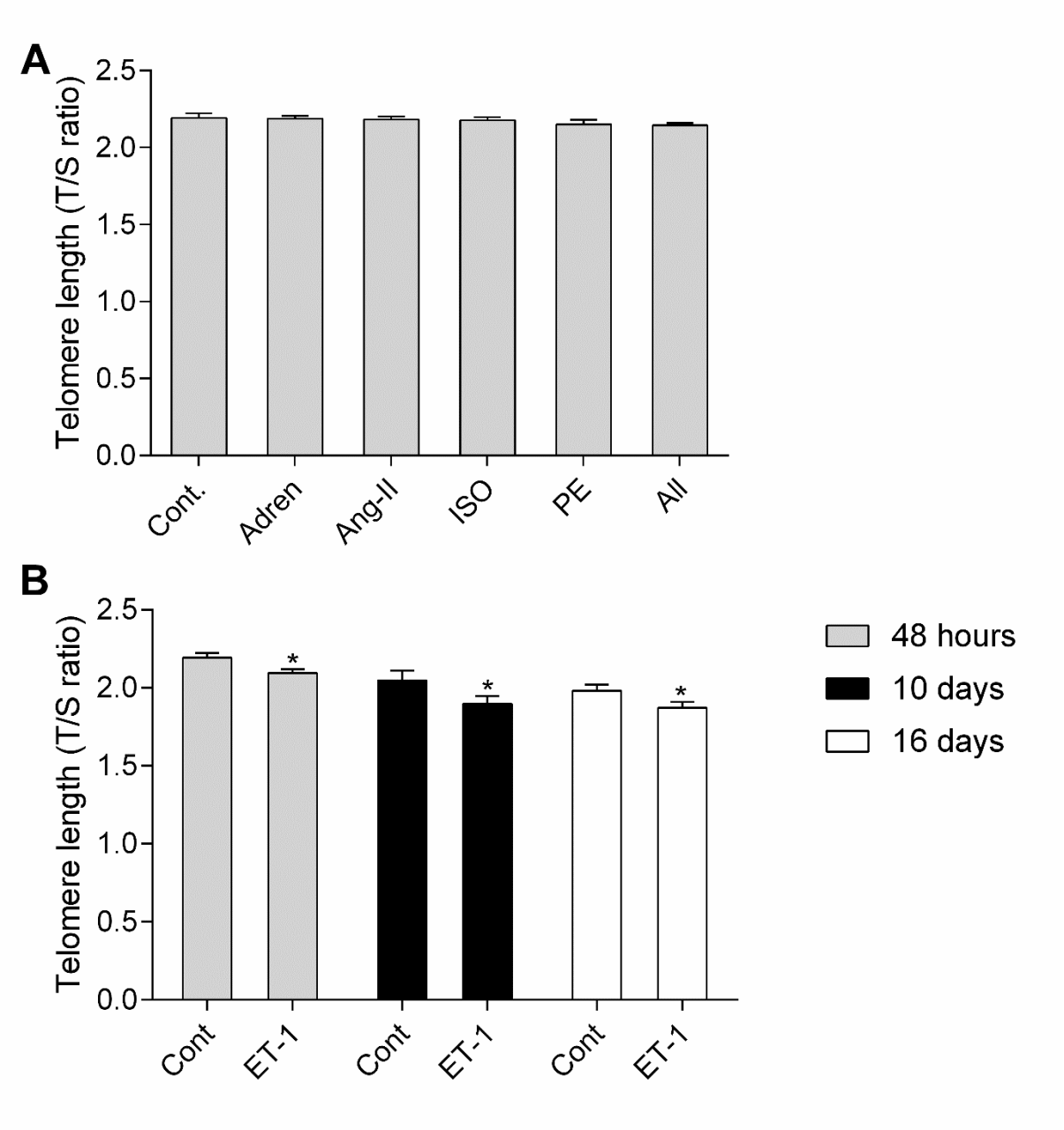

Figure 3. Telomere length. (A) Treatment with adrenaline, angiotensin-II, isoproterenol, phenylephrine, or all agonists for 48 hours did not affect telomere length; (B) Treatment with endothelin-1 shortened telomere length in a time-dependent manner. Data are presented as mean ± SD (n=3). * indicates $P<0.05$ compared to Cont. of same treatment duration. Cont, control; Adren, adrenaline; Ang-II, angiotensin-II; ET-1, endothelin-1; ISO, isoproterenol; PE, phenylephrine; All, all agonists. T/S ratio, telomere to single-copy gene ratio.

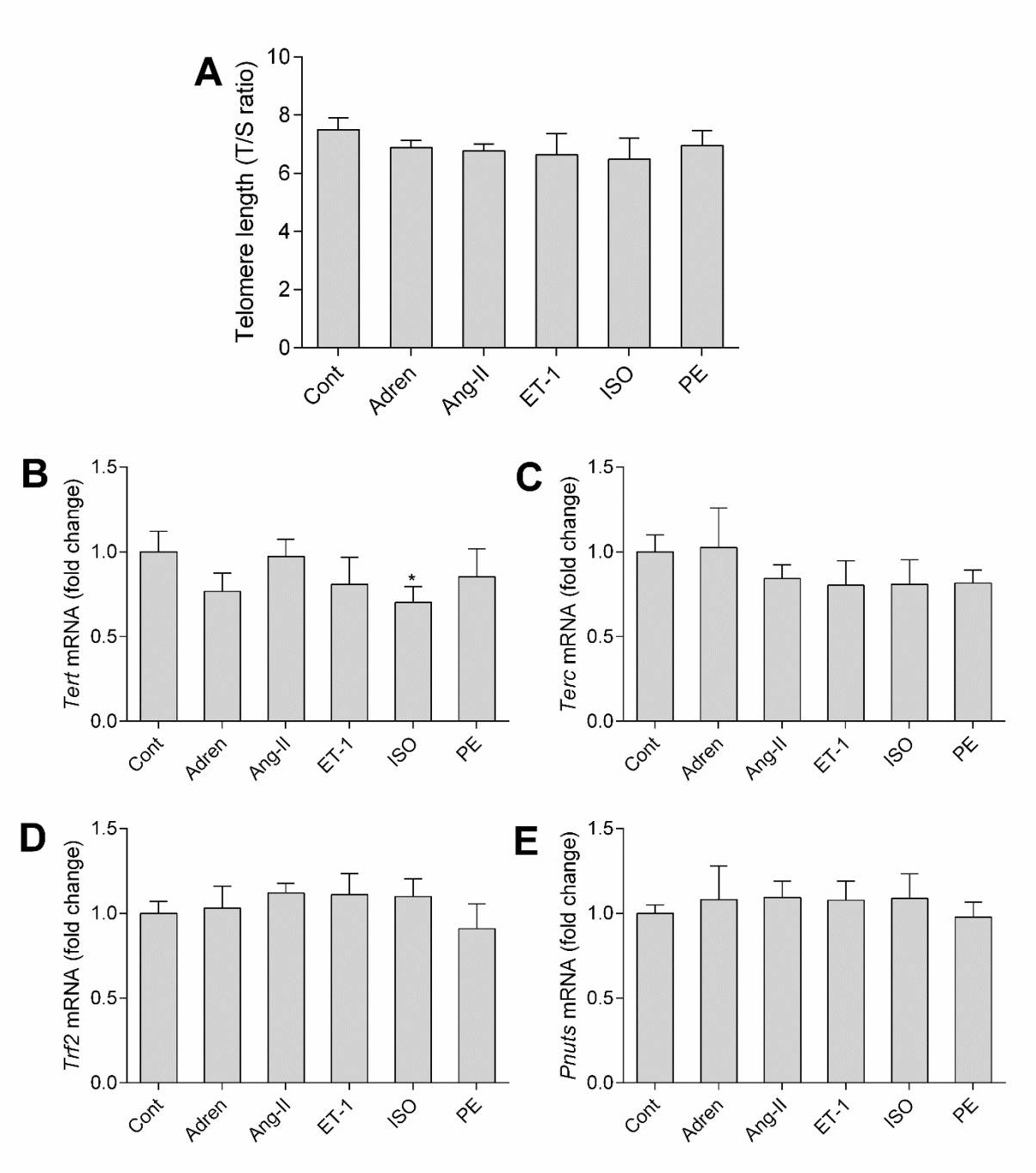

Figure 4. H9c2 telomere length and telomere-associated genes. (A) Inducing hypertrophy did not affect telomere length; (B) Isoproterenol treatment decreased H9c2 cell *Tert* mRNA levels; (C) Inducing hypertrophy did not affect *Terc*, (D) *Trf2*, or (E) *Pnuts* mRNA levels. All H9c2 cells. Data are presented as mean ± SD (n=3) with Cont normalised to 1. * indicates $P<0.05$ compared to Cont. Cont, control; Adren, adrenaline; Ang-II, angiotensin-II; ET-1, endothelin-1; ISO, isoproterenol; PE, phenylephrine. T/S ratio, telomere to single-copy gene ratio; *Tert*, telomerase reverse transcriptase; *Terc*, telomerase RNA component; *Trf2*, telomere repeat-binding factor 2; *Pnuts*, phosphatase nuclear-targeting subunit 1.

Telomerase gene expression

In HPCs, all agonists except Adren increased telomerase RNA component (*TERC*) mRNA levels ($P>0.05$) (Figure 5A). In contrast, however, there were no significant changes in telomerase reverse transcriptase (*TERT*) expression in any of the groups (Figure 5B). In H9c2 cells, *Tert* mRNA levels were significantly decreased following ISO treatment ($P=0.025$) (Figure 4B). Unlike the HPCs, there were no significant changes in *Terc* expression in any of the H9c2 cell groups (Figure 4C).

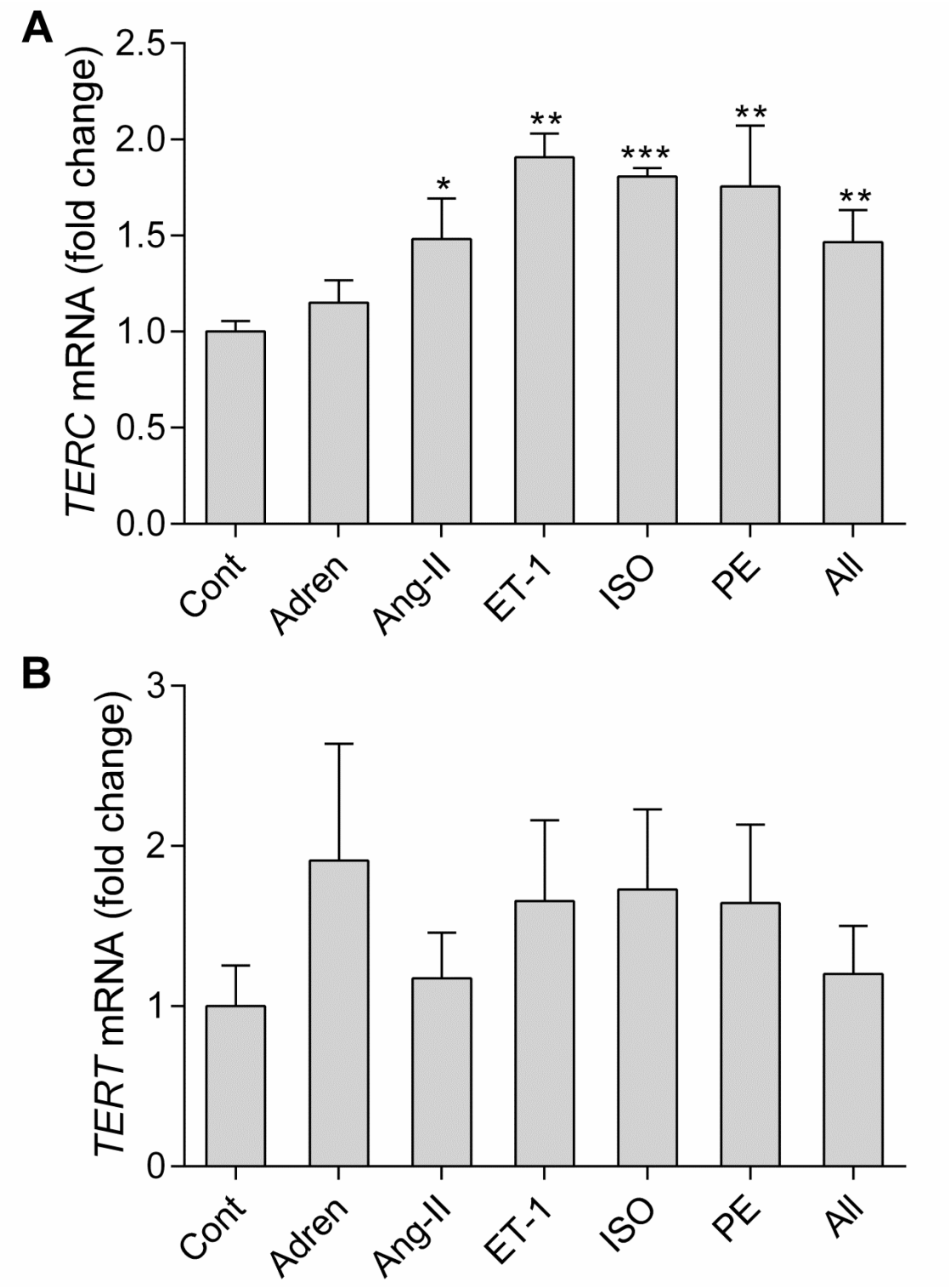

Figure 5. Telomerase gene expression. (A) Inducing hypertrophy increased *TERC* but not (B) *TERT* expression in HPCs. Data are presented as mean ± SD (n=3) with Cont. normalised to 1. * indicates $P<0.05$, ** $P<0.01$, and ** $P<0.001$ compared to Cont. Cont, control; Adren, adrenaline; Ang-II, angiotensin-II; ET-1, endothelin-1; ISO, isoproterenol; PE, phenylephrine. *TERT*, telomerase reverse transcriptase; *TERC*, telomerase RNA component.

Telomeric gene expression

In HPCs, telomere repeat-binding factor 1 *(TRF1)* expression was upregulated as a result of ISO treatment (P=0.003) (Figure 6A). Similarly, TRF1 interacting nuclear factor 2 (*TINF2*) mRNA levels were higher in ISO (P=0.010) as well as Ang-II (P=0.030) and ET-1 (P=0.010) groups (Figure 6B). In contrast, however, there were no significant changes in telomere repeat-binding factor 2 (*TRF2*) expression in any of the groups (Figure 6C). ISO significantly increased TRF2-interacting protein (*TERF2IP*) mRNA levels (P=0.011) (Figure 6D). Furthermore, both ISO and PE significantly upregulated tripeptidyl peptidase 1 (*TPP1*) (Figure 6E) and protection of telomeres 1 (*POT1*) expression (all P>0.05) (Figure 6F). Finally, sirtuin 1 (*SIRT1*) was significantly upregulated following ISO treatment (P=0.015) (Figure 6G) but there were no changes in protein phosphatase 1 regulatory subunit 10 (also known, and hereafter referred to, as *PNUTS*) expression in any of the groups (to Figure 6H). There were also no changes in *Trf2* (Figure 4D) or *Pnuts* (Figure 4E) mRNA levels in H9c2 cells.

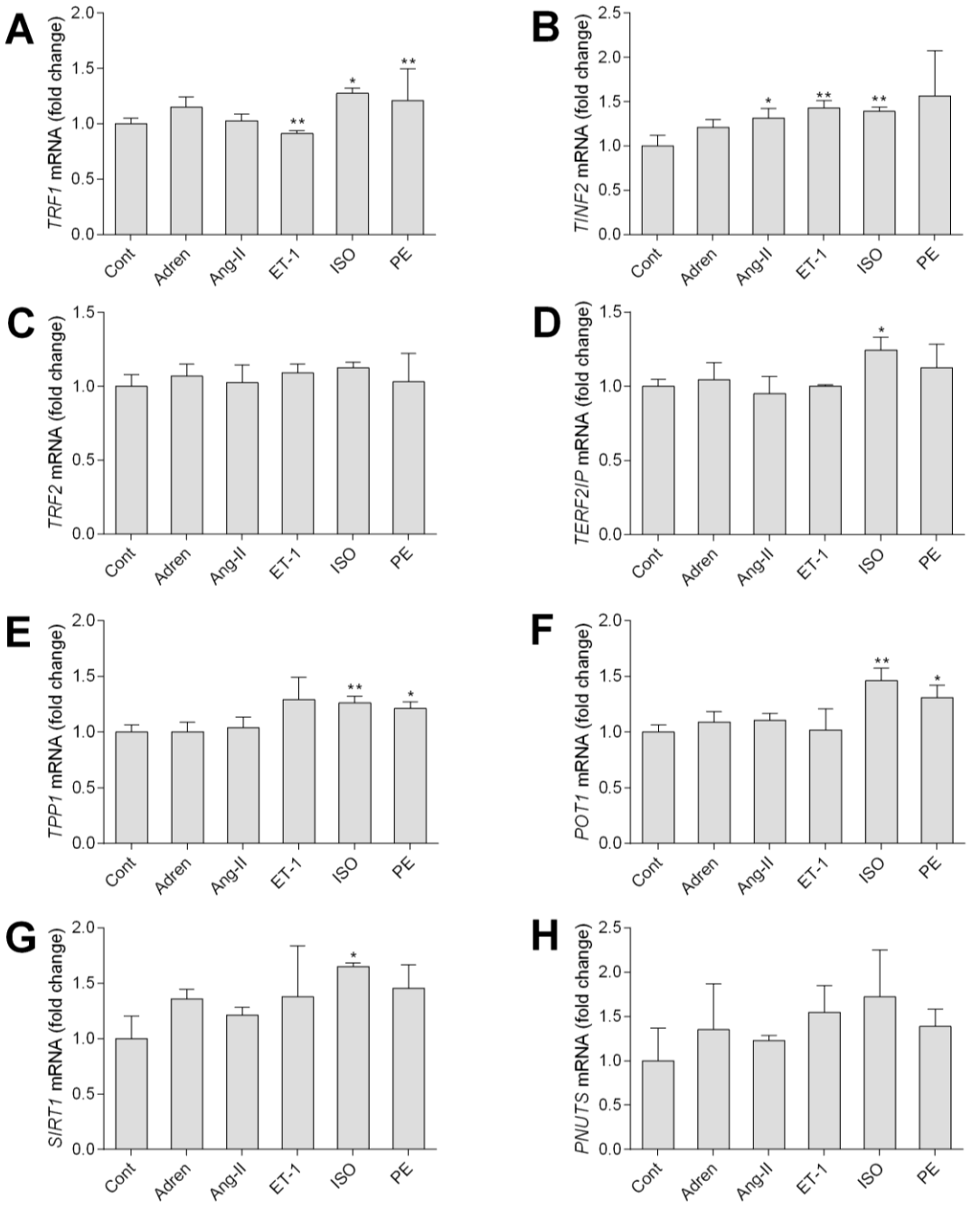

Figure 6. Telomeric gene expression. (A) Endothelin-1, isoproterenol, and phenylephrine increased *TRF-1* mRNA levels; (B) Angiotensin-II, endothelin-1, and isoproterenol upregulated *TINF-2* expression; (C) Inducing hypertrophy did not affect *TRF2* mRNA levels; (D) Isoproterenol increased *TERF2IP* expression; (E) Isoproterenol and phenylephrine increased *TPP1* and (F) *POT1* mRNA levels; (G) isoproterenol upregulated *SIRT1* expression; (H) Agonist stimulation did not affect *PNUTS* mRNA levels. All HPCs. Data are presented as mean ± SD (n=3) with Cont. normalised to 1. * indicates $P<0.05$ and ** $P<0.01$compared to Cont. Cont, control; Adren, adrenaline; Ang-II, angiotensin-II; ET-1, endothelin-1; ISO, isoproterenol; PE, phenylephrine. *TRF1*, telomere repeat-binding factor 1; *TRF2*, telomere repeat-binding factor 2; *TINF2*, TRF1 interacting nuclear factor 2; *TERF2IP*, TRF2-interacting protein; *TPP1*, adrenocortical dysplasia homolog; *POT1*, protection of telomeres 1; *PNUTS*, phosphatase nuclear-targeting subunit 1; *SIRT1*, sirtuin 1.

Markers of pathological and physiological cardiac hypertrophy

In HPCs, the gene for atrial natriuretic peptide (*NPPA*) was not significantly dysregulated in any treatment groups (Figure 7A). Expression levels of the brain natriuretic peptide (*NPPB*) were significantly downregulated in Adren (P=0.048) and PE (P=0.035) groups relative to the control (Figure 7B). Gene expression of the pro-hypertrophic and pro-inflammatory nuclear factor NF-kappa-B p105 subunit (*NFKB1*) was significantly upregulated in all treatment groups besides ET-1 (P<0.05) (Figure 7C). The gene for monocyte chemotactic protein 1 (*MCP-1*) was down regulated in AngII (P<0.001) and ET-1 (P=0.004) groups (Figure 7D). Mechanistic target of rapamycin (*MTOR*) was significantly upregulated after ET-1 (P=0.027), ISO (P=0.024), and PE (P=0.021) stimulation (Figure 7E). Phosphokinase C alpha (*PKCα*) was also upregulated after ISO (P=0.010) and PE (P=0.049) treatment (Figure 7F).

Markers of physiological cardiac hypertrophy were also significantly upregulated in HPCs. For instance, mitogen-activated protein kinase 1 (*MAPK1*) expression was significantly higher in all treatment groups (P<0.05) (Figure 7G). Furthermore, there was a trend towards increased expression of Protein Kinase AMP-Activated Catalytic Subunit Alpha 2 (*PRKAA2*) in all treatment groups but only PE (P=0.047) reached significance (Figure 7H). In contrast, there were no significant changes in *Mapk1* expression after agonist stimulation in H9c2 cells (Figure 8A).

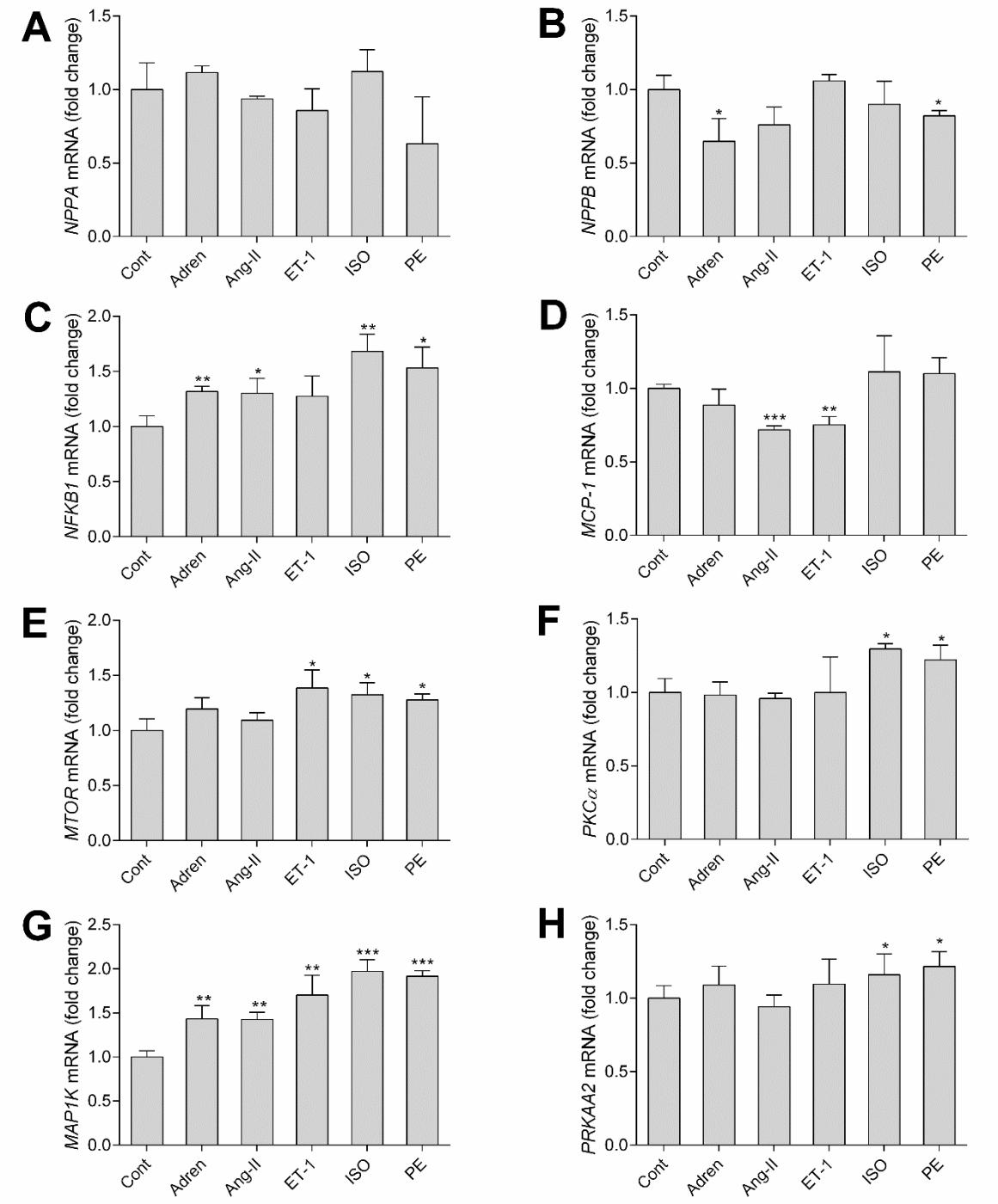

Figure 7. Markers of pathological and physiological cardiac hypertrophy. Agonist stimulation increased markers of pathological (A-F) and physiological (G-H) cardiac hypertrophy. All HPCs. Data are presented as mean ± SD (n=3) with Cont. normalised to 1. * indicates $P<0.05$, ** $P<0.01$, and ** $P<0.001$ compared to Cont. Cont, control; Adren, adrenaline; Ang-II, angiotensin-II; ET-1, endothelin-1; ISO, isoproterenol; PE, phenylephrine. *NPPA* gene for atrial natriuretic peptide; *NPPB*, gene for Brain natriuretic peptide; *NFKB1*, Nuclear factor NF-kappa-B p105 subunit; *MCP-1*, Monocyte chemotactic protein 1; *PKCα,* phosphokinase C alpha. *MTOR*, Mechanistic target of rapamycin; *MAPK1*, Mitogen-activated protein kinase 1; *PRKAA2*, Protein Kinase AMP-Activated Catalytic Subunit Alpha 2.

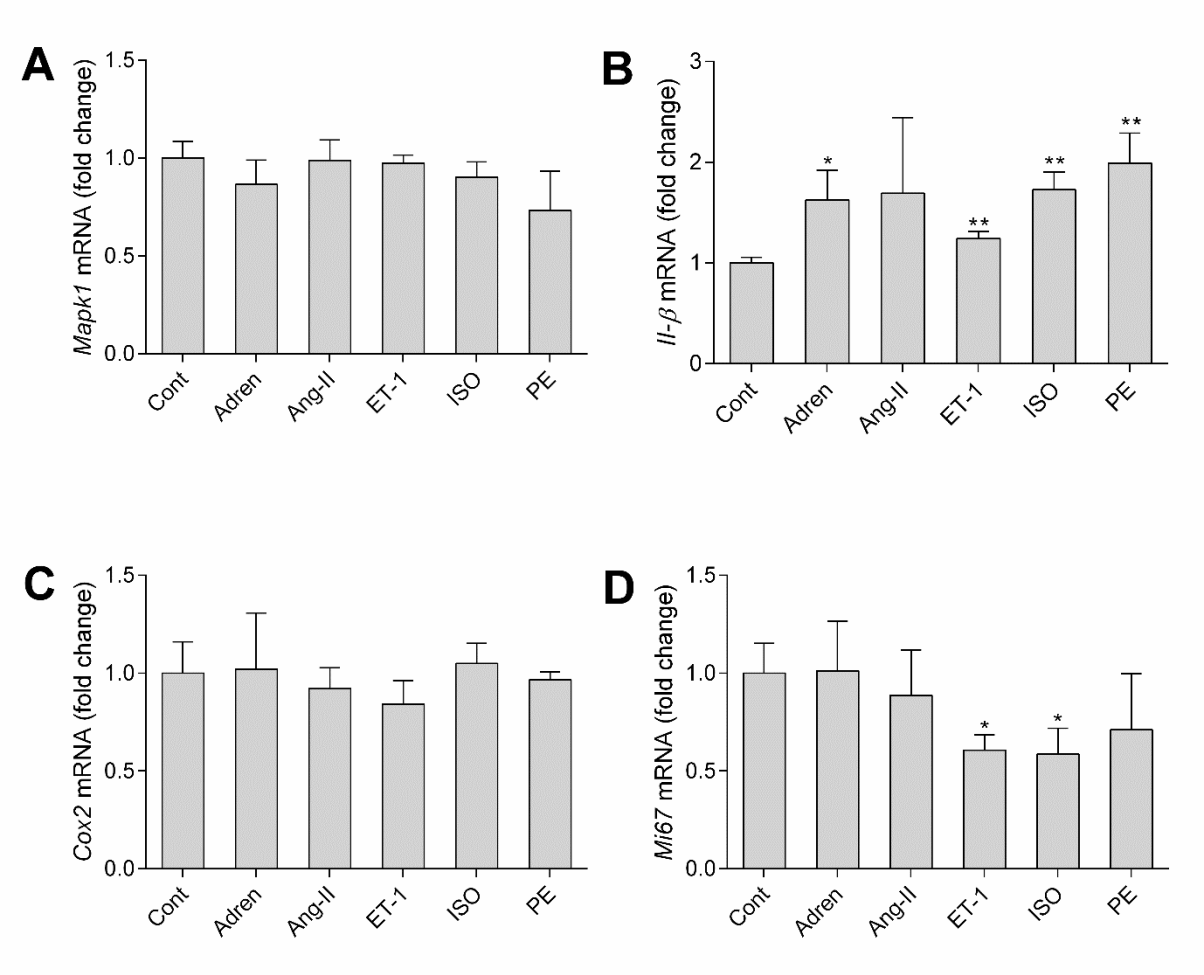

Figure 8. Expression of selected genes in H9c2 cells. These genes were selected for comparison because were dysregulated in HPCs. (A) Inducing hypertrophy did not affect *Mapk1* mRNA levels; (B) Adrenaline, endothelin-1, isoproterenol, and phenylephrine increased *Il-1β* expression; (C) Inducing hypertrophy did not affect *Cox2* mRNA levels; (D) Endothelin-1 and isoproterenol decreased *Mi67* expression. All H9c2 cells. Data are presented as mean ± SD (n=3) with Cont. normalised to 1. * indicates $P<0.05$ and ** $P<0.01$compared to Cont. Cont, control; Adren, adrenaline; Ang-II, angiotensin-II; ET-1, endothelin-1; ISO, isoproterenol; PE, phenylephrine. *Mapk1*, Mitogen-activated protein kinase 1; *Il-1β*, Interleukin-1β; *Cox2*, cyclooxygenase-2; *Mi67*, gene for Ki67.

Inflammation and oxidative stress

Interleukin 6 (*IL6*) expression was significantly downregulated ($P<0.05$) (Figure 9A) while interleukin-1β (*IL-1β*) mRNA levels were significantly upregulated ($P<0.05$) in all treatment groups (Figure 9B). Similarly, all agonists increased cyclooxygenase-2 (*COX2*) expression ($P<0.05$) (Figure 9C). In H9c2 cells, *Il-1β* was also significantly upregulated in all groups besides Ang-II ($P<0.05$) (Figure 8B) but there were no changes in *Cox2* mRNA levels (Figure 8C). Live cell imaging of HPCs revealed that reactive oxygen species (ROS) were significantly higher in Ang-II and ET-1 treated groups (Figure 9D and E).

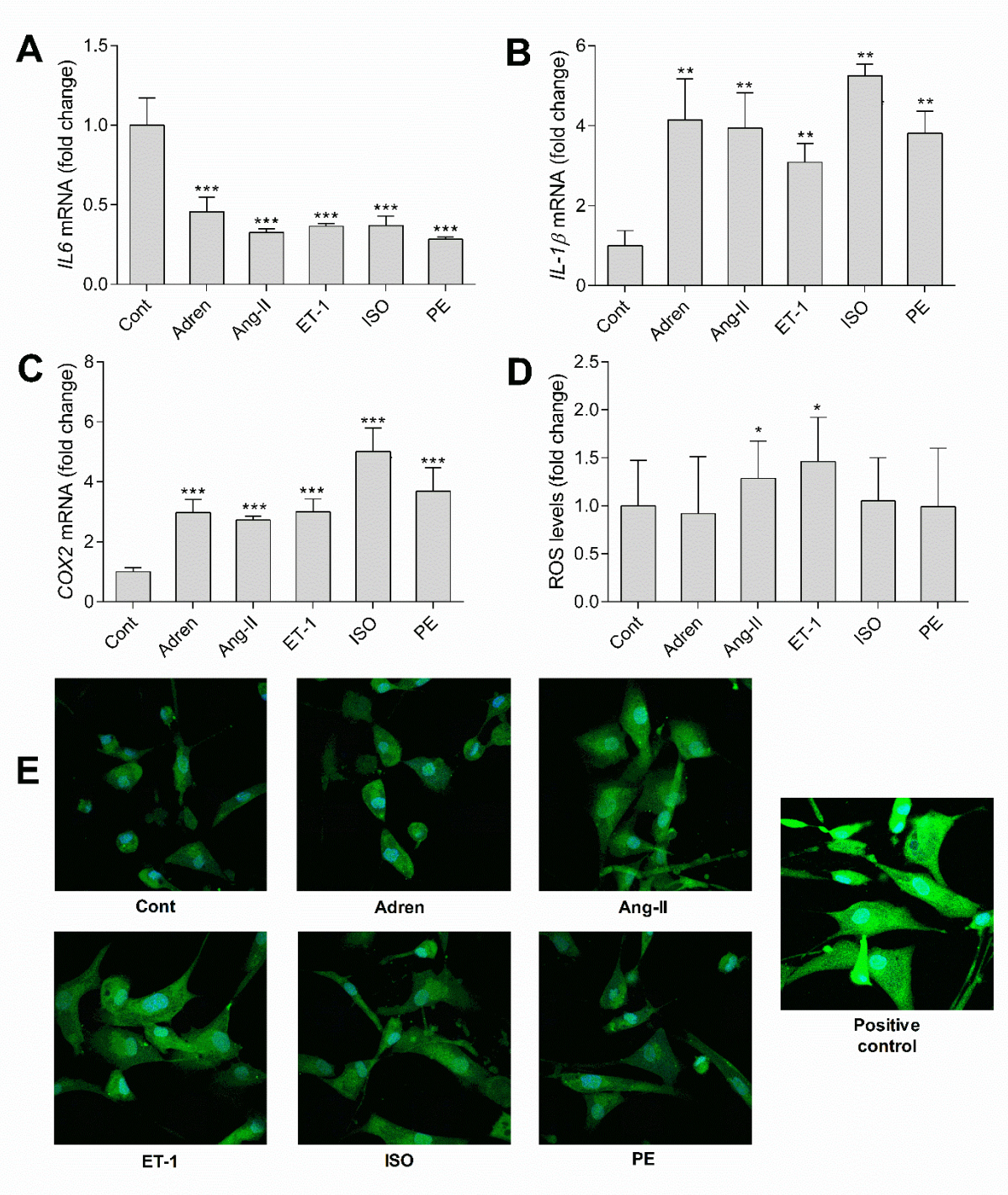

Figure 9. Inflammation and oxidative stress. (A) Inducing hypertrophy decreased *IL6* mRNA levels; (B) Inducing hypertrophy increased *IL-1β* expression; (C) Inducing hypertrophy increased *COX2* mRNA levels; (D) Angiotensin-II and endothelin-1 increased ROS levels; (E) 6-carboxy-2',7'-dichlorodihydrofluorescein diacetate immunofluorescence for ROS. All HPCs. Data are presented as mean ± SD (n=3 for gene expression and n=25 for ROS) with Cont. normalised to 1. * indicates $P<0.05$, ** $P<0.01$, and ** $P<0.001$ compared to Cont. Cont, control; Adren, adrenaline; Ang-II, angiotensin-II; ET-1, endothelin-1; ISO, isoproterenol; PE, phenylephrine. *IL6*, Interleukin 6; *IL-1β*, Interleukin-1β; *COX2*, cyclooxygenase-2; ROS, reactive oxygen species.

Proliferation and apoptosis

The nuclear antigen and marker of cardiomyocyte proliferation, Ki67 and its gene *Mi67*, were used to determine levels of proliferation. In HPCs, all agonists significantly increased Mi67 expression relative to the control ($P<0.05$) (Figure 10A). Ki67 antigen expression was also higher in all groups but only Adren ($P=0.047$), Ang-II ($P=0.040$), and ET-1 ($P=0.007$) reached significance (Figure 10B and C). In contrast, there was a significant decrease in *Mi67* expression following ET-1 ($P=0.013$) and ISO ($P=0.029$) treatment in H9c2 cells (Figure 8F). Immunoblotting of cleaved and native caspase 3 isoforms did not detect apoptosis in any of the HPC groups (Figure 10D).

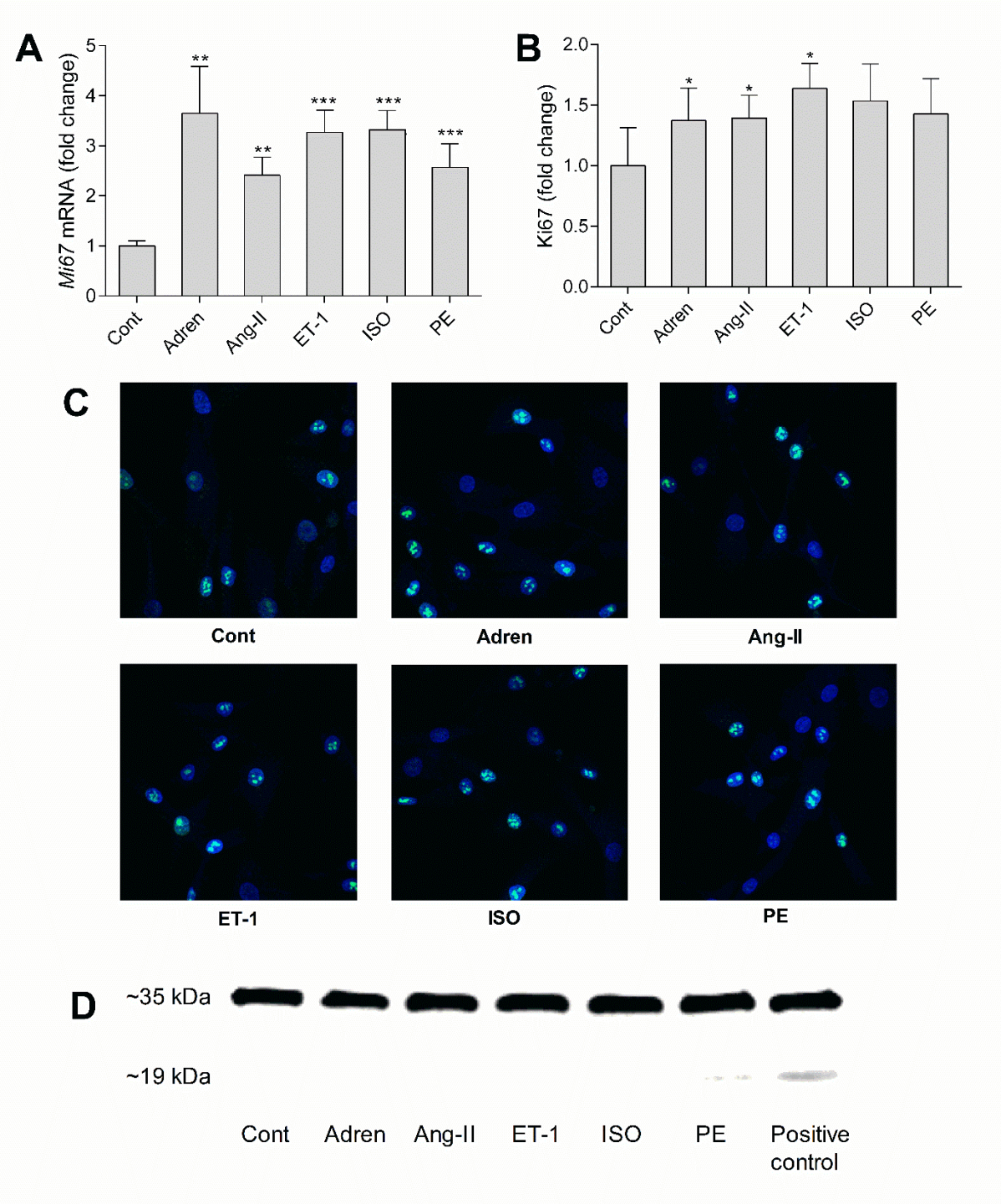

Figure 10. Proliferation and apoptosis. (A) Inducing hypertrophy increased *Mi67* mRNA levels; (B) Adrenaline, angiotensin-II, and endothelin-1 increased Ki67 antigen expression; (C) Anti-Ki67 immunocytochemistry; (D) Western blot of cleaved caspase 3. All HPCs. Data are presented as mean ± SD (n=3 for gene expression and n=7 for Ki67 immunocytochemistry) with Cont. normalised to 1. * indicates $P<0.05$, ** $P<0.01$, and ** $P<0.001$ compared to Cont. Cont, control; Adren, adrenaline; Ang-II, angiotensin-II; ET-1, endothelin-1; ISO, isoproterenol; PE, phenylephrine. *Mi67*, gene for Ki67.

Discussion

The present study is the first to directly assess the effects of cardiomyocyte hypertrophy on telomeres. Results demonstrated that all five agonists significantly increased HPC size after 48 hours of treatment but only ET-1 caused a reduction in telomere length. In addition, ET-1 treatment for 10 and 16 days continued to shorten telomeres. These findings point to an important and novel pathophysiological mechanism for ET-1 in human cardiac hypertrophy and aging. Importantly, this is independent of the vasoconstrictive and hypertensive properties typically attributed to ET-1. Agonist stimulation also increased H9c2 cell size but there were no significant changes in telomere length or the expression of selected genes that were dysregulated in the HPCs. This suggests that H9c2 cells do not accurately model human cardiomyocyte telomere dynamics *in vitro*.

Previous research has alluded to the possibility of ET-1 playing a role in cardiomyocyte aging. For instance, increased plasma concentrations of ET-1 have been observed in heart failure (49) and myocardial infarction (54), as have short cardiac telomeres (59, 74, 78, 82). Similarly, aging is associated with increases in ET-1 levels (53) and decreases in telomere length (7). Moreover, aerobic exercise interventions decrease ET-1 levels (43) and telomere attrition (42). On cardiomyocytes, there are two targets for ET-1 – ET_A (1) and ET_B receptors (67) – and ET-1 induces hypertrophy through the ET_A receptor (87). In contrast, Adren, ISO and PE activate α and/or β-ARs and Ang-II activates angiotensin type 1 and 2 receptors (92). Of these agonists, only Ang-II has been directly implicated in telomere shortening in cardiomyocytes (15) and other cell types (22). Interestingly, Ang-II can illicit ET-1 production (27, 39) and aging in the carotid arteries is dependent on ET-1 and not Ang-II (51). This suggests that ET-1 may play a role in the telomere attrition that has been attributed to Ang-II. Importantly, accelerated aging in human cardiomyocytes due to ET-1 and telomere shortening could have important consequences for

cardiac health as only young cells can produce functional progeny and replenish the heart (30).

One study suggested that the telomere attrition observed in cardiac hypertrophy and heart failure is likely a consequence of βAR-activation (65). Indeed, Raymond and colleagues found that daily ISO injections (0.02 mg/kg/day) for six months caused LV dilation and systolic chamber dysfunction in rats without affecting cardiac mass or telomere length (65). In the present study, ISO had a reliable influence on telomeric genes, upregulating six out of eight genes known to be associated with telomere length and structure which could, in part, explain the preservation of telomere length. In contrast to ISO treatments, there were few or no changes in telomeric gene expression in the other agonist groups. This demonstrates that cardiomyocyte hypertrophy can occur independently of changes in telomeres. Dysregulated telomeric gene expression, however, can be transient (11) so basal mRNA levels could be expected given there was 24 hours between the second agonist treatment and the extractions. *TERC* expression, however, was higher in all agonist groups besides Adren. Interestingly, an increase in *TERC* expression is cardio- and telomere-protective (81), suggesting this finding is likely to be a short-term compensatory mechanism, similar to the increase in telomerase activity observed in early-stage heart disease (36, 70, 75).

In contrast to telomeric genes, hypertrophic genes have been shown to peak 24-48 hours after agonist stimulation (71). In the present study, however, there were no increases in expression of the fetal genes and markers of heart failure *NPPA* and *NPPB* in any of the hypertrophic groups. Indeed, with the exception of ISO and PE increasing *PKCα* and *mTOR* mRNA levels, most gene expression findings reflected physiological rather than pathological cardiac hypertrophy. For instance, the cardioprotective genes *MAP1K* and *NFKB1* (85, 86) were significantly upregulated in all treatment groups (except for ET-1 in the latter). Although this suggests that acute agonist stimulation in HPCs may model physiological

rather than pathological cardiac hypertrophy, a transient increase in the concentration of hypertrophic agonists is often associated with cardio-protective activities. For instance, both plasma ET-1 (17) and Ang-II (83) levels have been shown to increase after exercise in healthy young males. Therefore, it may be unreasonable to expect short-term agonist treatments to reflect all the changes observed in degenerative and irreversible (i.e. pathological) cardiac hypertrophy.

Consistent with previous research (4, 35, 47), ROS was significantly upregulated following Ang-II and ET-1 treatment which could explain the telomere attrition observed in the ET-1 group. Furthermore, Ang-II is thought to contribute to the development of pathological hypertrophy in rodents by inducing oxidative stress (35, 89) and findings of the present study suggest this mechanism may also be involved in HPC hypertrophy. Interestingly, mice with short telomeres have increased endothelin-converting enzyme-1 (ECE-1) and thus synthesise more ET-1. Importantly, treatment with antioxidants reduces ECE-1 promoter activity and ET-1 levels (61). Telomerase-deficient cells also have increased ROS synthesis (61), further suggesting a link between ET-1 and ROS. Taken together, these findings demonstrate that ET-1 can induce ROS which may cause telomere shortening, accelerated cardiomyocyte aging, and cardiac hypertrophy. Given *Terc*$^{-/-}$ mice have increased ROS (61), it is possible that insufficient telomerase could catalyse and/or exacerbate this pathway.

Results of the present study also suggest an important role for inflammation in cardiomyocyte hypertrophy, with all agonists increasing *IL6* and *COX2* mRNA levels, as previously reported (38, 46). Inflammation is thought to be an important mediator of telomere length and may be the epiphenomenon linking heart failure and short telomere length (65). In support of this suggestion, ET-1 is pro-inflammatory in vascular smooth muscle cells (29). Furthermore, Ang-II-induced telomere attrition in human glomerular mesangial cells could be

partially reversed by losartan (a β-AR antagonist) treatment (22) which has been shown to reduce inflammation (and oxidative stress) (32). Increased inflammation was also the only finding observed in both the HPCs and H9c2 cells (besides increases in cell size). As H9c2 cells and primary neonatal rat cardiomyocytes show similar hypertrophic responses *in vitro* (80), this suggests that the inflammatory response may be highly conserved. However, given the lack of overall similarity in the expression of telomeric variables between HPCs and H9c2 cells, embryonic rat cells may not be appropriate models of human heart disease.

Like increased inflammation, another consistent finding of the present study was an increase in HPC proliferation following agonist stimulation (although this was observed only in HPCs and not H9c2 cells). Although Ki67 has been used as a marker of cardiomyocyte proliferation (31), it is also associated with cardiomyocyte ploidy (77) and thus may reflect changes in DNA content and other cell cycle activities rather than true cell division. Importantly, cardiomyocyte nuclear mitotic division is observed in late-stage heart failure, but this is accompanied by even higher rates of cardiomyocyte apoptosis (41). Therefore, measures of proliferation alone may not reflect an adaptive response as increased cell cycle activity in cardiomyocytes could also indicate a compensatory response to even higher levels of apoptosis. In this regard, however, no cleaved caspase 3 was detected in the present study, suggesting that the apoptotic rate did not increase in agonist-treated cells. Importantly, the increase in markers of proliferation and inflammation provide potential mechanisms through which agonist stimulation could lead to telomere attrition, as observed in late-stage cardiac hypertrophy and heart failure (59). In addition, data from the present study suggests that ET-1 may play a more direct role in cardiomyocyte telomere regulation, ROS levels, and thus viability (Figure 11).

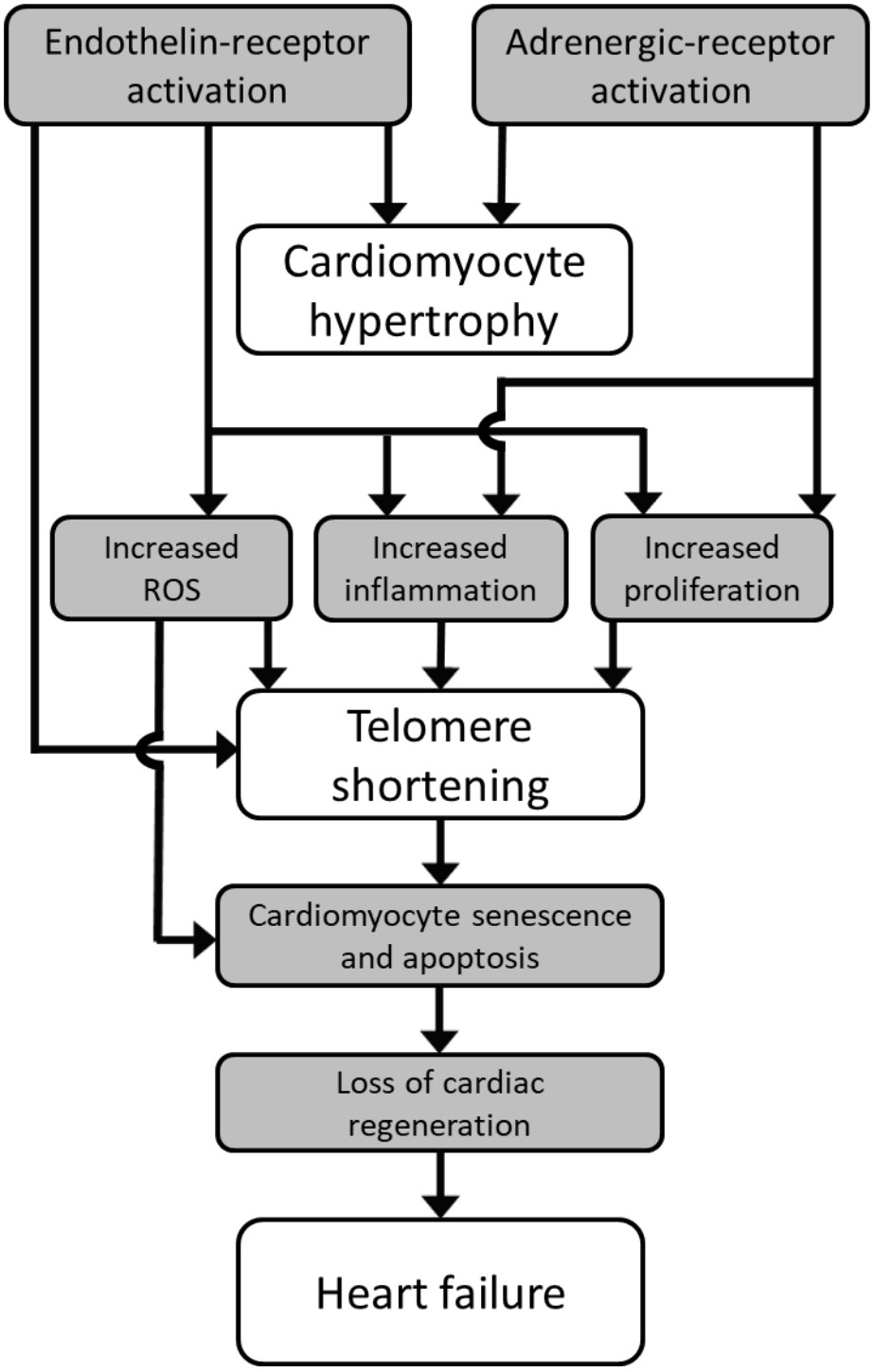

Figure 11. A simplified pathway of the proposed involvement of agonist-induced hypertrophy in cardiomyocyte telomere shortening, senescence, apoptosis, and heart failure. ROS, reactive oxygen species.

This study also has some limitations. Firstly, the use of immature human cardiomyocytes and immortalised rat cardiomyoblasts from homogenous populations fails to indicate if the changes reported here are universal. Secondly, the 48-hour timepoint may have missed acute transient changes in gene expression in the short-term and possible effects on telomere length and apoptosis in the long-term. Thirdly, while the *in vitro* approach allowed ET-1 and other agonists to be studied independently of vasoconstrictive (and thus biomechanical stress-inducing) affects, this leaves unanswered questions regarding interactions *in vivo*. Future studies could utilise selective $ET_{A/B}$ agonists or antagonists to elucidate the mechanism of action of ET-1 on telomeres and perform rescue treatments with recovery times from acute treatments and anti-inflammatory or antioxidant agents to preserve cardiomyocyte telomeres and function. This would help elucidate the involvement of telomeres in the development of cardiomyocyte hypertrophy and the mechanisms governing the transition into degenerative heart failure.

In conclusion, results of the present study highlight a novel pathophysiological mechanism of ET-1 in cardiomyocyte aging and viability. These findings also demonstrate that changes in telomere length or telomeric gene expression are not necessary for AR-induced increases in cardiomyocyte size. In this regard, the lack of changes in gene expression suggest that inflammation, proliferation, and oxidative stress may play crucial roles in cardiomyocyte telomere attrition. The inconsistent expression of hypertrophic genes highlights the need for more time points to uncover novel and reliable factors that are directly involved in the pathophysiology of cardiac hypertrophy leading to heart failure and could be targeted for therapeutic use. Finally, changes to telomeric variables following hypertrophy in HPCs are not reflected in H9c2 cells, highlighting important differences between human and rat cardiomyocyte aging and viability.

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Chapter 5

The Effects of Inhibiting Telomere Maintenance on Human Cardiomyocyte Size

Abstract

Background and aims: Telomeres are nucleoprotein repeats that protect chromosome ends and prevent the cellular senescence and apoptosis. The number of these repeats (telomere length) is negatively associated with cardiomyocyte hypertrophy, a key feature of heart disease, but this relationship is poorly defined. The present study aims to characterise key aspects of the molecular and cellular phenotype of human primary cardiomyocytes (HPCs) after inhibiting telomere maintenance.

Methods and results: HPCs were treated with small interfering RNA (siRNA) knockdowns of TERT, the catalytic component of the telomere-lengthening enzyme telomerase, and TRF2, an integral telomeric protein, and the small molecule telomerase inhibitor BIBR1532. Cell size, proliferation, apoptosis, and oxidative stress were measured by confocal microscopy. Telomere length and gene expression were determined using qPCR. HPC size significantly increased following siRNA knockdown of *TERT*, *TRF2*, both *TERT* and *TRF2*, and inhibition of telomerase activity for 48 hours (all $P<0.001$). Telomere length, however, only decreased in cells treated with BIBR1532 for ten days ($P=0.016$). Oxidative stress ($P=0.010$), apoptosis ($P=0.001$), and expression of the hypertrophic genes *NPPA* and *NPPB* (both $P<0.001$) increased following *TRF2* knockdown while all treatments reduced proliferation ($P<0.05$) and expression of the cardioprotective gene *MAPK1* ($P<0.05$).

Discussion and conclusions: These findings demonstrate that disruptions in telomere maintenance are sufficient to induce cardiomyocyte hypertrophy independently of changes telomere length. The involvement of TRF2 in maintaining telomere loops suggests an important role for the telomeric structure in regulating hypertrophic gene expression and maintaining cardiomyocyte viability.

Introduction

Telomeres are hexanucleotide TTAGGG repeats which combine with specialised proteins at the ends of eukaryotic chromosomes (11). The primary role of telomeres is to protect genomic content and prevent cellular senescence and apoptosis (10). To achieve this, sufficient telomere length must be maintained and telomeric proteins available to form the protective loops that encapsulate the genome (30). As such, several studies have measured telomere length and maintenance in search for mechanisms that regulate cell loss in aging and degenerative diseases (38, 39, 110). In this regard, there is a well-documented association between telomere attrition and heart disease, the worldwide leading cause of death (18). There is also significant evidence, however, linking short telomeres to old-age (13), obesity (108), diabetes (110), and other causes of heart disease (3). This overlap of primary risk factors has prevented researchers from identifying if the relationship between telomeres and heart disease is causal, consequential, or confounding.

The cell-specific length of telomeres (33) and the multi-organ pathophysiology of many diseases, such as hypertension (26) and atherosclerosis (63), can also pose problems to uncovering causal relationships. Cardiac hypertrophy, however, is caused by the growth of individual cardiomyocytes, the contractile cells of the heart, in response to biomechanical stress or other hypertrophic stimuli (44, 107). Although this is harmless in its early stages, continued hypertrophy of the myocardium progressively decreases the size of the left ventricle and, consequently, cardiac output (35). As such, unchecked cardiac hypertrophy leads to heart failure (85), a disease which is characterised by cardiomyocyte-specific telomere shortening (81). Furthermore, cardiomyocyte telomere length is negatively associated with cell size in mouse hearts at various ages (78). Therefore, the involvement of telomeres in the pathophysiological sequence of cardiac hypertrophy leading to heart failure is contained to the cardiomyocytes. As such, changes to cardiomyocyte telomeres in relation

to cell size could uncover a causal relationship between telomeres, cardiac hypertrophy, and heart failure.

The length of telomeres is determined by heritable differences (91), cellular replicative history (8), endogenous stresses (58), environmental toxins (3), and other factors (39). Consequently, telomeres shorten progressively throughout the lifespan but can be elongated by telomerase, an enzyme comprised of a reverse transcriptase (Tert) and an RNA component (Terc) (66). In mice deficient of either *Tert* or *Terc*, cardiomyocytes lose 3-5 kilo bases (kb) of telomeric repeats per generation and early-onset cardiac hypertrophy and heart failure occur by the second and fifth generations, respectively (15). Although this suggests a causal involvement for telomeres in cardiac hypertrophy and heart failure (90), systemic telomerase knockdowns cause hypertension and increased levels of the pro-hypertrophic peptide endothelin-1 (73) both of which can lead to cardiac hypertrophy (44). This confounds the direct relationship between telomeres and the development of cardiac hypertrophy, limiting the utility of these models.

Another shortcoming of animal studies are the differences in the length and maintenance of telomeres. For instance, critical telomere length associated with senescence in rodents is around 15 kb (78) whereas in humans it is only 1.5 kb (97). Furthermore, *Tert* overexpression prevents the terminal differentiation of cardiomyocytes in neonatal mice (67) but has no influence on the senescence of human fetal cardiomyocytes (5). In this regard, fetal or neonatal cardiomyocytes are often used in the study of cardiac hypertrophy but have many key differences when compared to adult cells (18). For instance, the so-called fetal genes *NPPA* and *NPPB* are essential for cardiac development but their re-expression in adulthood is associated with cardiac hypertrophy and heart failure (102). Furthermore, a decrease in cardiac telomere length and telomerase activity is normal around birth (19, 96) but predisposes to heart disease in adulthood (25, 68). Similarly, immortalised cell cultures

are also used in cardiac research (18) but the circumvention of telomere-induced senescence and apoptosis seriously questions their relevance in the study of degenerative diseases. The final factor to consider is that cardiac hypertrophy can also be a physiological response to strenuous exercise (36). Although this is potentially confounding, physiological cardiac growth is associated with increased telomere length and maintenance (54, 101) as well as the expression of specific genes, such as mitogen activated protein kinase 1 (*MAPK1*) and protein kinase AMP-activated catalytic subunit alpha 2 (*PRKAA2*) (61). These findings suggest that telomere length and maintenance are important epigenetic factors in determining whether cardiac growth is normal, adaptive, or degenerative.

The specialised proteins that bind to the telomeric sequence and form the so-called shelterin complex prevent telomeres from being recognised as DNA breaks (31). Of these, telomere repeat-binding factor 2 (TRF2) has been shown to be crucial for cardiomyocyte viability. For instance, interference with *Trf2* in rat cardiomyocytes causes telomere shortening, decreased proliferation, and apoptosis whereas high levels of *Trf2* are protective (68). Moreover, unlike telomerase-deficient mice, which are healthy in the F1 and F2 generations (90), *Trf2* knockouts are embryonically lethal (57). Interestingly, *Trf2* deletion in the mouse liver did not impair liver regeneration or mouse survival (49), highlighting cell-specific functions for *Trf2*. In humans, *TRF2* insufficiency predisposes cardiomyocytes to apoptosis (17) and is associated with cardiomyocyte telomere attrition and heart failure (68). Taken together, these findings suggest an important role for *TRF2* in the development of heart failure. However, the effects of *TRF2* knockdown on cardiomyocyte size have not been determined.

Given the involvement of telomere length and maintenance in the development of cardiac hypertrophy and subsequent heart failure, the present study used small-interfering RNAs (siRNAs) to knockdown the expression of *TERT* and *TRF2* in adult human primary

cardiomyocytes (HPCs) and then measure cell size. Telomerase activity was also inhibited pharmacologically in HPCs with the small molecule BIBR1532, as no studies have determined the effects of telomerase inhibition on cardiomyocytes without the use of genetic manipulation. The aim of this study was to identify molecular factors that characterise cardiac hypertrophy and heart failure after inhibiting telomere maintenance to determine the possibility of a causal relationship. It was hypothesised that inhibiting telomere maintenance would lead to an increase in cardiomyocyte size.

Methods

Ethical clearance

As this study used a cell line, no ethical approval was necessary.

Cell culture

HPCs from a healthy 56-year-old Caucasian male were acquired commercially and cultured in ready-to-use myocyte growth medium (both from Promo Cell, Heidelberg, Germany). Although HPCs are derived from adult cardiac progenitor cells and replicated for at least 15 population doublings *in vitro*, they also undergo senescence and normal cellular aging (95, 105) and thus more accurately reflect human disease than immortalised cell lines and animal models. Passage 4 HPCs were seeded at 25% confluence, and allowed to adhere for 24 hours before treatment. 6-well dishes were used for nucleic acid and protein extraction and single glass-bottom dishes of the same size (Mattek Corporation, Ashland, USA) for imaging. All HPCs were passaged using HEPES wash buffer, myocyte trypsin, and myocyte trypsin inhibitor, and stored in myocyte cryo-media (all from Promo Cell).

Cell treatments

siRNA transfection

HPCs were transfected with small interfering RNAs (siRNAs) for 48 hours to reduce TERT (Bioneer #1149877) and TRF2 (Bioneer #1149866) mRNA levels. A scramble siRNA (Bioneer #SN-1002) with no known binding regions in the human genome was used as a control. Each siRNA was diluted in Opti-MEM Reduced Serum Media (Life Technologies) to a concentration of 10nM and delivered using RNAimax Lipofectamine (Life Technologies). This concentration has been used previously in muscle cells (65) and early optimisations showed it produce at least a 50% knockdown of target gene expression in HPCs

(data not shown). A double transfection of TERT and TRF2 was also performed as research has suggested an interplay between these genes in the heart (47, 67).

Pharmacological inhibition of telomerase activity

Genetic methods of interfering with telomeric gene expression may decrease the presence of crucial components at telomeres and lead to telomere uncapping (76). Telomerase inhibition with small molecules, however, such as 2-((E)-3-naphthalen-2-yl-but-2-enoylamino)-benzoic acid, known as BIBR1532, may simply decrease telomerase activity without disturbing the structure of telomeres (23). Therefore, to inhibit telomerase activity without genetic interference, HPCs were treated with 930nM of the human telomerase inhibitor BIBR1532 (Merck Millipore) for 48 hours. This concentration was selected as it lies between the lower (28) and higher (6) estimates of the half-maximal inhibitory concentration (IC_{50}) values previously reported for HeLa cells.

Despite telomerase activity being the only mechanism of elongating, and thus maintaining, telomere length in normal cells (66), changes in telomere length take time to occur, even in the absence of telomerase activity (12, 15, 51). To confirm the efficacy of BIBR1532 to reduce HPC telomere length, and not simply induce changes in telomere structure, 930nM treatment was continued on a subset of HPCs every 48 hours for ten days. Cells were passaged on days two and six and only telomere length was determined in this group.

Telomere length

HPCs were scraped from two wells with a rubber policeman into 200uL of PBS and Genomic DNA was extracted using the Purelink Genomic DNA Mini Kit (Life Technologies) according to the manufacturer's protocol and diluted to 2.5ng/µL based on quantitation with a Nanodrop 2000. This was performed using three independent to provide enough data for

statistical analyses. A 10ng template (4µL) was then added to a 384-well quantitative polymerase chain reaction (qPCR) plate (Life Technologies) and telomere length was determined using the telomere (T) to single-copy gene (S) method as described previously (22). Briefly, the squared cycle threshold (Ct) for 36B4, a single-copy gene ('S') present only once in the genome, was divided by the square of the Ct for Tel1 ('T'), which reflects telomere length. This provides a measure of average telomere length, known as the T/S ratio. Primers, reagents, qPCR equipment, and cycling conditions were the same as used in Chapter 5 and previously published (24).

Gene expression

RNA was isolated using 1mL of TRI reagent (Life Technologies) per 3 wells. Cell lysates were frozen at -80°C then extracted at a later date in accordance with the manufacturer's guidelines. Immediately following extraction, the concentration of RNA was determined using a Nanodrop 2000 and 2µg of RNA was reverse transcribed into cDNA using the High Transcriptase cDNA kit (Life Technologies) as instructed by the manufacturers. This was performed on three independent replicates to provide enough data for statistical analyses. cDNA samples were diluted to 5ng/uL and the same reagents, qPCR equipment, and cycling conditions as per Chapter 5 and previous research (24) were used. Primers design was achieved with NCBI tool Primer Blast (http://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi) to ensure all primers spanned at least on exon-exon junction, produced an amplicon of 75-150 base pairs in length, and had a melting temperature as close to 60°C as possible. Key genes involved in telomere maintenance (7, 29, 31, 55), pathological (20, 27, 40, 45, 59) and physiological (21, 80, 109) cardiac hypertrophy, inflammation (87, 103), and proliferation (43) were measured relative to the housekeeping gene β-actin (93). Primer sequences were the same as those used and listed in Chapter 5 (see pages 155-157).

Cell size

HPCs size was demonstrated by immunocytochemistry (ICC) and z-stack confocal imaging. Briefly, cells were washed (in PBS) twice, fixed in 4% paraformaldehyde for 30 minutes, washed four more times, blocked and permeabilised in 0.75% 2mg/mL bovine serum albumin, 2% horse serum, and 0.4% Tx100 in PBS for 30 minutes, then probed with an anti-α-sarcomeric actin monoclonal antibody (Sigma Aldrich) 1:100 overnight at 4°C. Following two washes, the AP130C Goat Anti-Mouse IgG & IgM Antibody Cy3 conjugate (Merck Millipore) 1:100 was applied for two hours in the dark at room temperature. Cells were then washed twice, 2μM of Hoechst 33342 in PBS was added for 5 minutes, and four final washes were performed before imaging in PBS. 1μm slices were captured over a 15μm z-range using a Nikon Eclipse Ti-E confocal microscope. A minimum of 25 cells within five z-stacks were analysed per group using integrated density measurements generated in ImageJ.

Proliferation

To measure levels of proliferation, siRNA-treated cells were prepared for ICC and confocal microscopy as described above then probed with a conjugated antibody for Ki67 (Abcam), a nuclear antigen and marker of cardiomyocyte proliferation (9), 1:100 overnight at 4°C. Cells were washed twice and 2μM of Hoechst 33342 in PBS was added for 5 minutes before four final washes and imaging in PBS. Eight sections containing 6-10 cells were captured on a Nikon Eclipse Ti-E confocal microscope. Proliferation was determined by dividing the number of cells expressing the Ki67 antigen by the total number of cells per image.

Apoptosis

Immunocytochemistry and confocal microscopy

Cells were prepared as above then probed for cleaved caspase 3 (Cell Signalling #9664) 1:100 overnight at 4°C to determine levels of apoptosis. Prior to this, a subset of HPCs were

treated with 500nM staurosporine (Sigma Aldrich) for 3 hours to induce apoptosis and act a positive control. Following two washes, the AP130C Goat Anti-Mouse IgG & IgM Antibody Cy3 conjugate (Merck Millipore) 1:100 was applied for two hours in the dark at room temperature. Cells were then washed twice, 2μM of Hoechst 33342 in PBS was added for 5 minutes, and four final washes were performed before imaging in PBS. Five z-stacks consisting of one image every μm over a 15μm z-range were captured using a Nikon Eclipse Ti-E confocal microscope. Levels of apoptosis was defined as the integrated density of total cleaved caspase 3 fluorescence per cell generated in ImageJ.

Protein extraction

Protein samples were collected from 3 wells using 300uL of RIPA buffer (Sigma Aldrich) with 1% Halt Protease & Phosphatase Inhibitor Cocktail (Thermo Scientific) and then stored at -80ºC. Total soluble protein was extracted as per the manufacturer's instructions then immediately diluted 1:3 and measured with the bicinchoninic acid (BCA) assay kit (Bio-Rad). A standard curve with eleven points ranging from 0.0 – 2.0 mg/mL was created using bovine serum albumin (BSA) (Thermo Scientific) and the absorbance at 570nm was determined with a Multiskan Microplate Photometer (Thermo Scientific).

Western blot

25μg of cell protein was mixed with 5% β-Mercaptoethanol (Sigma Aldrich) in Laemelli Sample Buffer (Bio-Rad) then denatured at 100°C for 5 minutes. Samples were resolved on a 4–15% Mini-PROTEAN® TGX™ Precast Gel (Bio-Rad) at 80 volts for around 90 minutes at room temperature then electroblotted on to a Nitrocellulose Membrane (Thermo Scientific) at 100 volts for one hour at 4°C. The membrane was blocked for one hour at room temperature using 5% skim milk in TBS-Tween 20 before poly (ADP-ribose) polymerase (PARP) (Cell signalling #9542) 1:2500 was applied overnight at 4ºC. PARP binds double

stranded DNA to mediate chromosomal stability (66) and becomes cleaved in cells undergoing apoptosis (69). The next morning, four 15-minute washes in TBS-Tween 20 were performed followed by the application of anti-rabbit (Cell signalling #7074) 1:5000 in a 0.5% skim milk in TBS-Tween 20 solution for 1 hour at room temperature. The wash steps were then repeated before detection using enhanced chemiluminescence SuperSignal West Pico Substrate (Thermo Scientific). Images were captured with a UVITEC Alliance digital imaging system (Thermo Scientific). The membrane was stripped for 15 minutes at room temperature using Restore^{TM} Western Blot Stripping Buffer (Thermo Scientific) then blocked in 5% skim milk in TBS-Tween 20. Cleaved PARP (Cell signalling #5625) (1:2500) was then applied and treated identically to PARP.

Reactive oxygen species

Oxidative stress was measured in live HPCs using the Image-iT LIVE Green Reactive Oxygen Species (ROS) Detection Kit (Life Technologies) and HPC reagents (Promo Cell) according to the manufacture's protocol. For a positive control, 100μM of tert-butyl hydrogen peroxide (TBHP), an inducer of ROS production (Life Technologies), was added to subset of cells for 2 hours. Single images were taken to avoid photobleaching on a Nikon Eclipse Ti-E confocal microscope system with a 37°C chamber. Total fluorescence was determined using ImageJ and a minimum of 25 cells were analysed per group.

Data Analysis

Statistical tests and data transformation were performed in Microsoft Excel and PRISM was used to create the graphs. For confocal microscopy and gene expression experiments, statistical analyses were performed on the least transformed relative data (the integrated density and delta Ct values, respectively) but graphed as a fold change for meaningful representation. Although all groups are displayed together for simplicity, with one graph per

dependent variable, each treatment group was compared to its control only using the appropriate *t*-test. The control for the *TERT* and *TRF2* knockdowns was the scramble siRNA and BIBR1532 was compared to a no treatment control group. Data are presented as means (M) ± the standard deviation (SD) with $P<0.05$ regarded as significant.

Results

Telomere length

48 hours of *TERT*, *TRF2*, or combined *TERT* and *TRF2* knockdown did not change HPC telomere length (all $P>0.05$) (Figure 1A). Similarly, BIBR1532 did not influence telomere length after 48 hours ($P=0.561$); however, there was a significant reduction in telomere length following 10 days of telomerase inhibition with BIBR1532 ($P=0.016$) (Figure 1B).

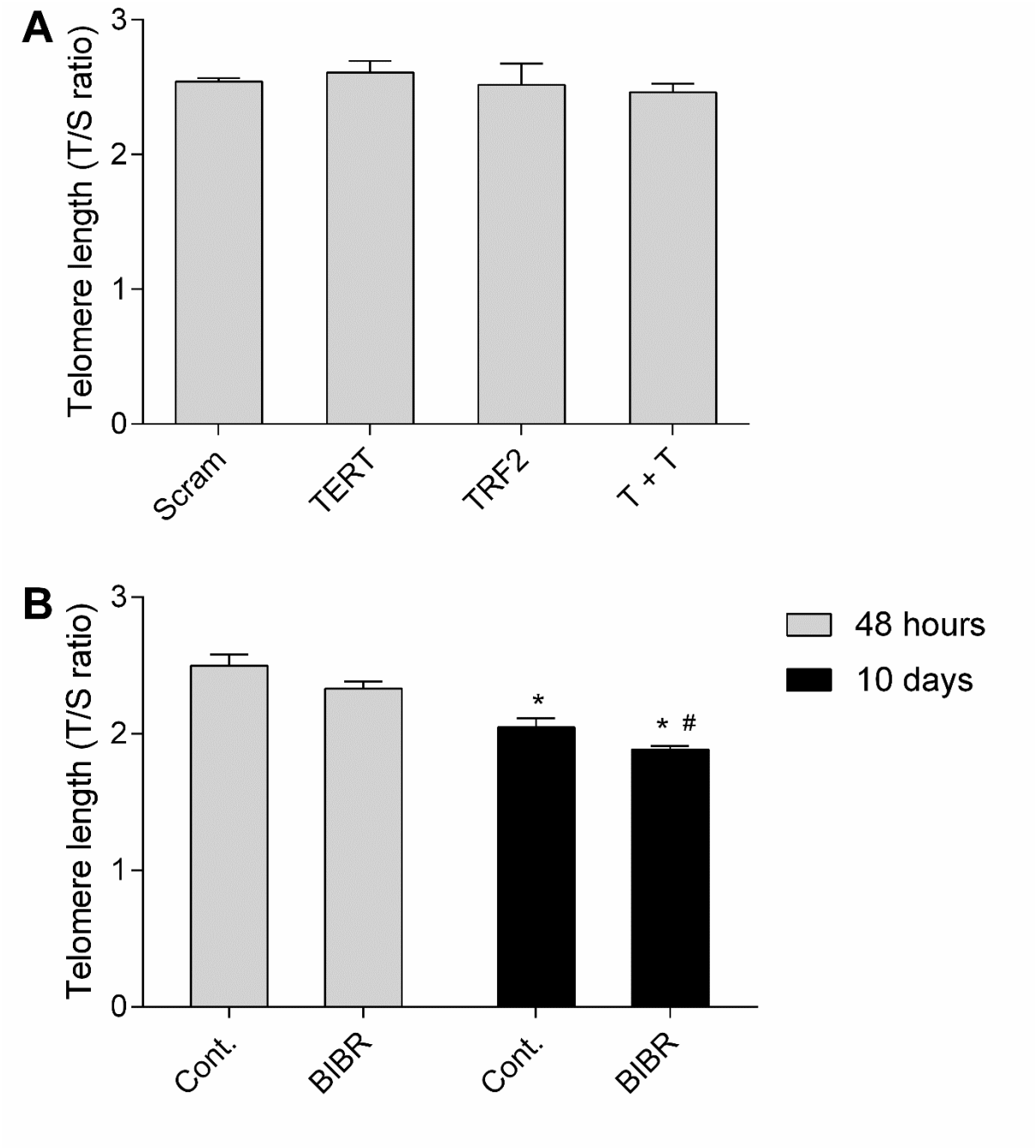

Figure 1. Telomere length. (A) HPC telomere length was unchanged after 48 hours but (B) shorter after 10 days of telomerase inhibition. Data are presented as mean ± SD (n=3). * indicates $P<0.05$ compared to 48-hour Cont. # indicates $P<0.05$ compared to 10-day Cont. Scram, Scramble siRNA; TERT, siRNA-TERT; TRF2, siRNA-TRF2; T + T, siRNA-TERT and siRNA-TRF2; Cont., no treatment control; BIBR, BIBR1532.

Validation of knockdowns

TERT-siRNA knockdown significantly decreased *TERT* mRNA levels (P=0.039) but not when used in conjunction with the *TRF2*-siRNA (P=0.638) (Figure 2A). *TRF2* knockdown produced a significant decrease in *TRF2* expression when used independently (P=0.011) and combined with the *TERT*-siRNA (P=0.010) (Figure 2B). BIBR1532 treatment did not alter *TERT* or *TRF2* mRNA levels (both P>0.05) (Figure 2A and B, respectively).

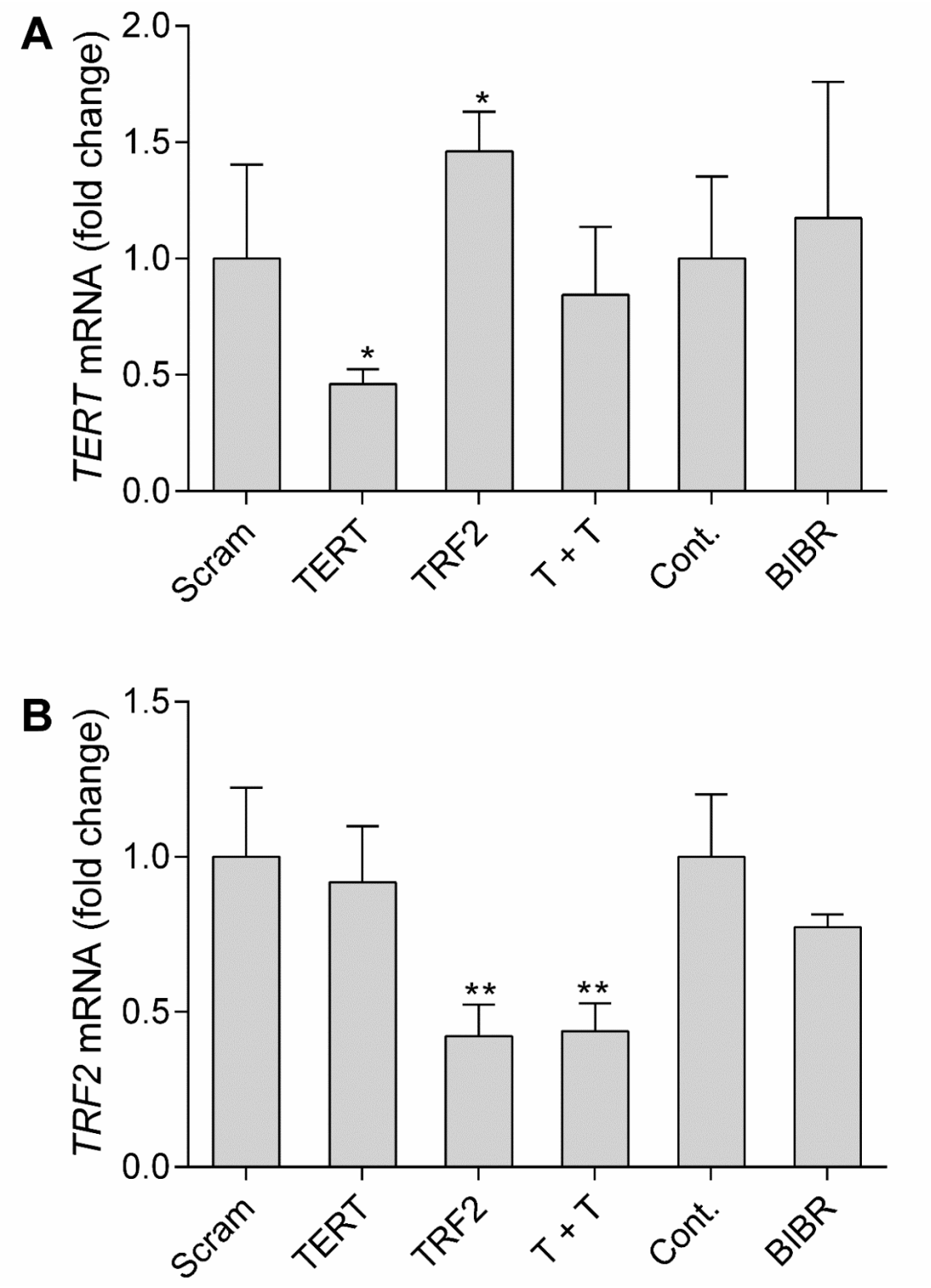

Figure 2. Validation of knockdowns. (A) *TERT* and (B) *TRF2* mRNA levels were decreased by targeted siRNA knockdown but not telomerase inhibition in HPCs. Data are presented as mean ± SD (n=3) with Scram normalised to 1. * indicates $P<0.05$ compared to Scram. * indicates $P<0.05$ compared to Scram. ** indicates $P<0.01$ compared to Scram. Scram, Scramble siRNA; TERT, siRNA-TERT; TRF2, siRNA-TRF2; T + T, siRNA-TERT and siRNA-TRF2; Cont., no treatment control; BIBR, BIBR1532.

Cell size

Targeted siRNA-knockdown of *TERT*, *TRF2*, and *TERT* and *TRF2* combined, all significantly increased HPC size relative to the scramble control ($P<0.001$) (Figure 3). Treatment with the telomerase inhibitor BIBR1532 also significantly increased HPC size compared to untreated control cells ($P<0.001$) (Figure 3).

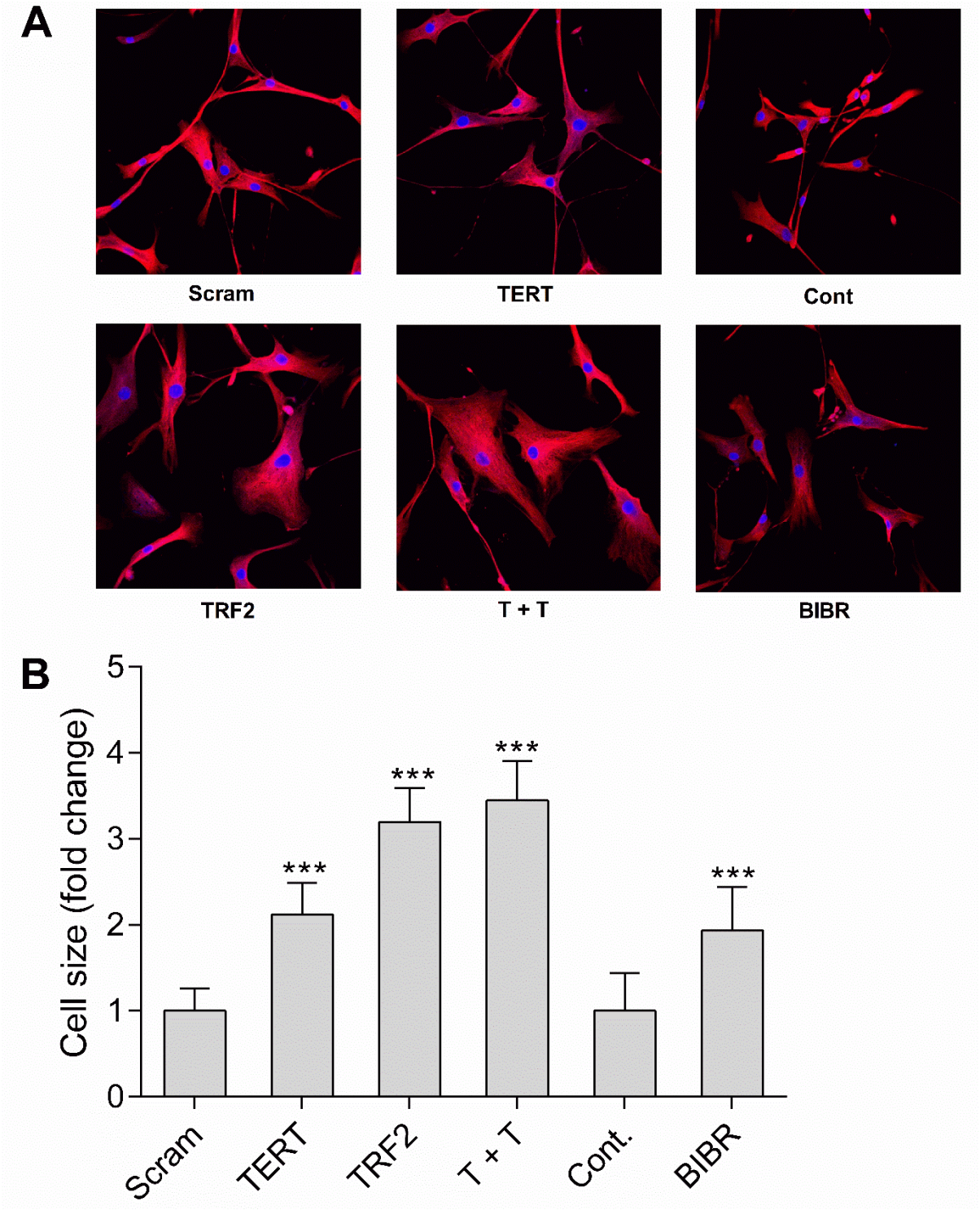

Figure 3. Cell size. (A) Anti-α-sarcomeric actin immunocytochemistry; (B) inhibiting telomere maintenance increased HPC size. Data are presented as mean ± SD (n=25) with Scram and Cont normalised to 1. *** indicates $P<0.001$ compared to Scram or Cont. Scram, Scramble siRNA; TERT, siRNA-TERT; TRF2, siRNA-TRF2; T + T, siRNA-TERT and siRNA-TRF2; Cont., no treatment control; BIBR, BIBR1532.

Telomeric gene expression

siRNA inhibition of *TERT* caused a significant reduction in *TERC* expression, independently (*P*=0.019) and in conjunction with *TRF2* (*P*<0.001) (Figure 4A). Telomerase inhibition decreased mRNA levels of two key shelterin components, telomere repeat-binding factor 1 (*TRF1*) (*P*=0.015) (Figure 4B), and TRF1 interacting nuclear factor 2 (*TINF2*) (*P*=0.016) (Figure 4C). None of the treatments influenced the expression of the genes for the shelterin proteins TRF2-interacting protein (*TERF2IP*) (Figure 4D) or protection of telomeres 1 (*POT1*) (both *P*>0.05) (Figure 4E). Combined *TERT* and *TRF2* knockdown decreased mRNA levels of Tripeptidyl-peptidase 1 (*TPP1*), another shelterin component, (*P*=0.014) (Figure 4F) and protein phosphatase 1 regulatory subunit 10 (also known, and hereafter referred to, as *PNUTS*), a telomeric gene involved in TRF2 binding (*P*=0.032) (Figure 4G). No treatments altered the expression of the lifespan modulator and telomere-associate gene Sirtuin 1 (*SIRT1*) (all *P*=0.05) (Figure 4H). *TRF2* knockdown had no effect on any of the above genes (all *P*>0.05).

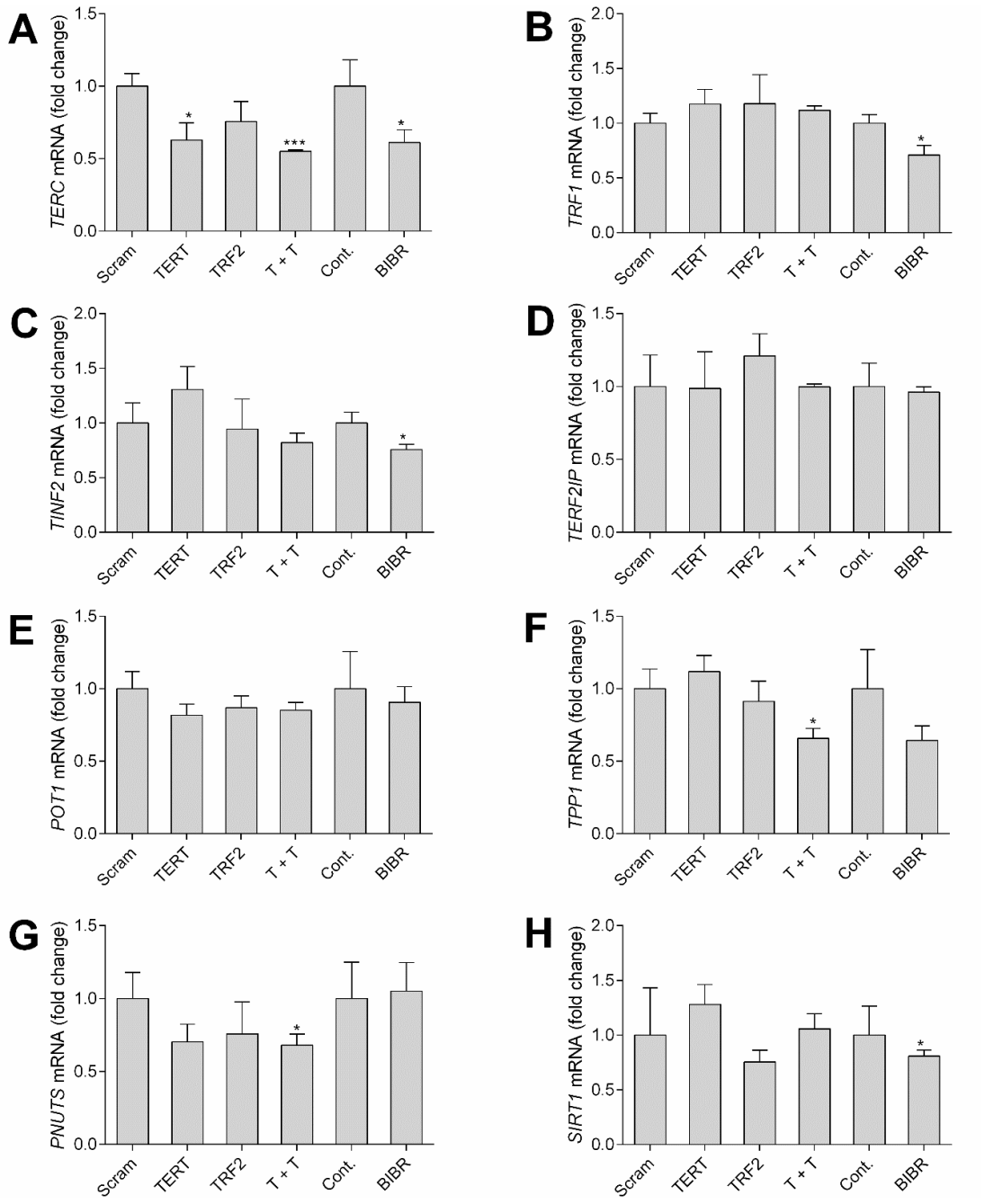

Figure 4. Telomeric gene expression. (A) *TERT* knockdown and BIBR1532 treatment reduced *TERC* mRNA levels; (B) BIBR1532 treatment decreased *TRF1* and (C) *TINF2* expression; (D) Inhibiting telomere maintenance did not affect *TERF2IP* or (E) *POT1* mRNA expression; (F) Combined *TERT* and *TRF2* knockdown decreased *TPP1* and (G) *PNUTS* mRNA levels; (H) BIBR1532 treatment decreased *SIRT1* expression. All HPCs. Data are presented as mean ± SD (n=3) with Scram and Cont normalised to 1. * indicates $P<0.05$ compared to Scram or Cont. *** indicates $P<0.001$ compared to Scram or Cont. Scram, Scramble siRNA; TERT, siRNA-TERT; TRF2, siRNA-TRF2; T + T, siRNA-TERT and siRNA-TRF2; Cont., no treatment control; BIBR, BIBR1532. *TRF1*, telomere repeat-binding factor 1; *TRF2*, telomere repeat-binding factor 2; *TINF2*, TRF1 interacting nuclear factor 2; *TERF2IP*, TRF2-interacting protein; *TPP1*, tripeptidyl peptidase 1; *POT1*, protection of telomeres 1; *PNUTS*, phosphatase nuclear-targeting subunit 1; *SIRT1*, Sirtuin 1.

Markers of pathological and physiological cardiac hypertrophy

TRF2 knockdown significantly increased expression of the fetal genes and markers of heart failure *NPPA* and *NPPB*, both independently, and in conjunction with *TERT* (all $P<0.001$) (Figure 5A and B respectively). mRNA levels of NF-kappa-B p105 subunit (*NFKB1*), a gene associated with pathological cardiac hypertrophy (40) were not changed in any of the groups (Figure 5C) (all $P>0.05$). Expression of phosphokinase C alpha (*PKCα*), another gene associated with pathological cardiac hypertrophy (45) increased as a result of *TERT*-siRNA treatment ($P=0.048$) (Figure 5D). Inhibition of telomerase activity with BIBR1532 had no effect on the expression of any of the above genes (all $P>0.05$). Similarly, no treatments altered the gene expression of protein kinase AMP-activated catalytic subunit alpha 2 (*PRKAA2*), a gene associated with physiological cardiac growth (all $P>0.05$) (Figure 5E). In contrast, all treatments significantly decreased expression of the cardioprotective gene *MAPK1* (all $P<0.05$) (Figure 5F).

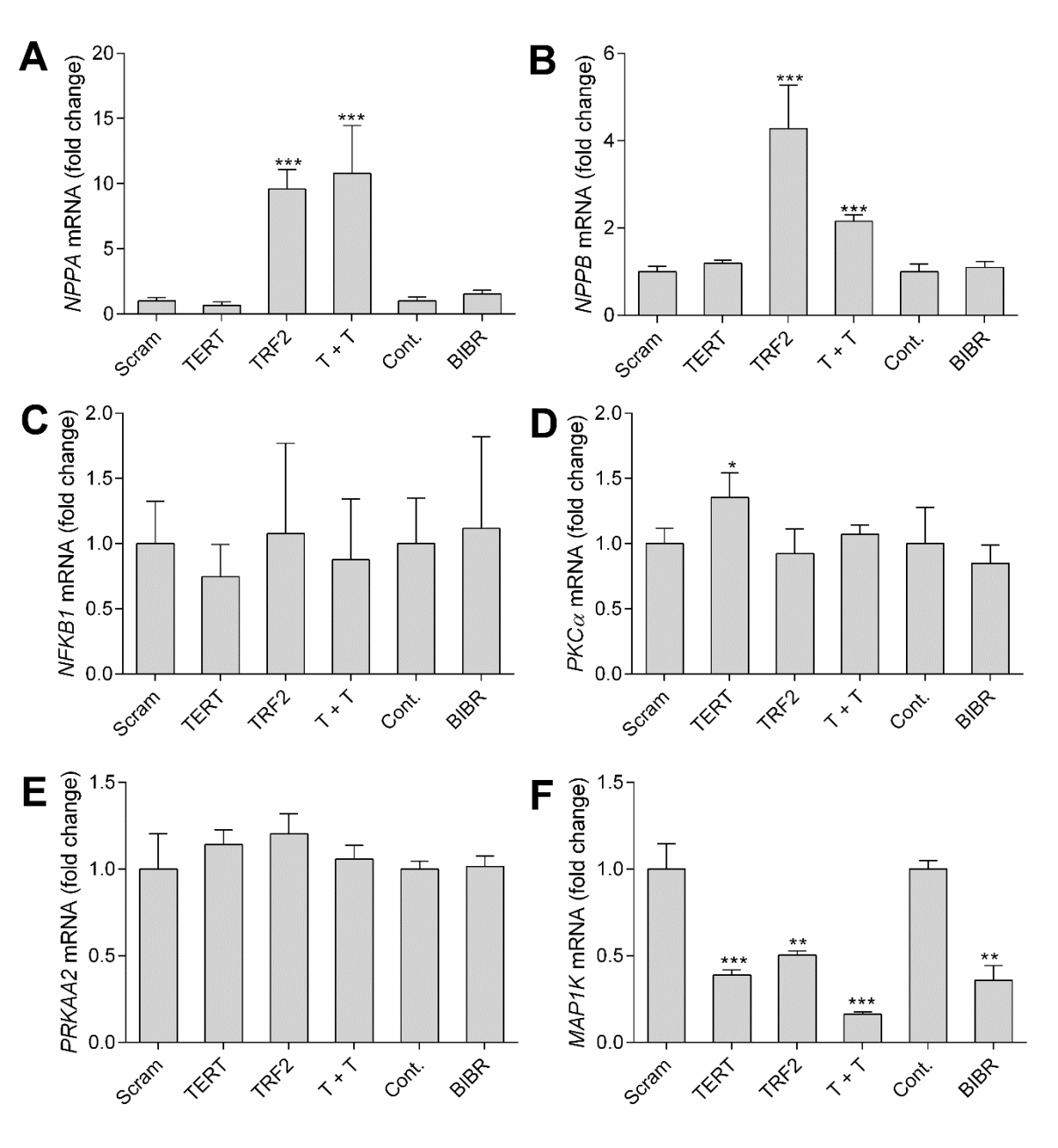

Figure 5. Markers of pathological and physiological cardiac hypertrophy. (A) *TRF2* knockdown increased *NPPA* and (B) *NPPB* expression; (C) Inhibiting telomere maintenance did not affect *NFKB1* mRNA levels; (D) *TERT* knockdown increased *PKCα* expression; (E) Inhibiting telomere maintenance did not affect *PRKAA2* mRNA levels; (F) Inhibiting telomere maintenance decreased *MAPK1* mRNA levels. All HPCs. Data are presented as mean ± SD (n=3) with Scram and Cont normalised to 1. * indicates $P<0.05$ compared to Scram or Cont. ** indicates $P<0.01$ compared to Scram or Cont. *** indicates $P<0.001$ compared to Scram or Cont. Scram, Scramble siRNA; TERT, siRNA-TERT; TRF2, siRNA-TRF2; T + T, siRNA-TERT and siRNA-TRF2; Cont, no treatment control; BIBR, BIBR1532. *NPPA* gene for atrial natriuretic peptide; *NPPB*, gene for brain natriuretic peptide; *NFKB1*, Nuclear factor NF-kappa-B p105 subunit; *MAPK1*, Mitogen-activated protein kinase 1; *PRKAA2*, Protein Kinase AMP-Activated Catalytic Subunit Alpha 2.

Inflammation and oxidative stress

As inflammation is a suspected cause of cardiac hypertrophy, heart failure, and telomere attrition (42, 58, 75), markers of inflammation were assessed. No treatments altered expression of the inflammatory markers interleukin 6 (*IL6*) and 1beta (*IL-β*) (all $P>0.05$) (Figure 6A and B, respectively). *TRF2* knockdown increased ($P=0.027$) and *TERT*-siRNA treatment decreased ($P=0.029$) expression of the marker of cardiomyocyte inflammation cyclooxygenase 2 (*COX2*) (Figure 6C).

Another cause of telomere attrition and cardiomyocyte apoptosis is oxidative stress (53, 99). Levels of ROS significantly increased following *TRF2*-siRNA treatment ($P=0.010$) but not when used in conjunction with the *TERT*-siRNA ($P>0.05$) (Figure 6D and E). The influence of BIBR1532 treatment on ROS levels was not assessed.

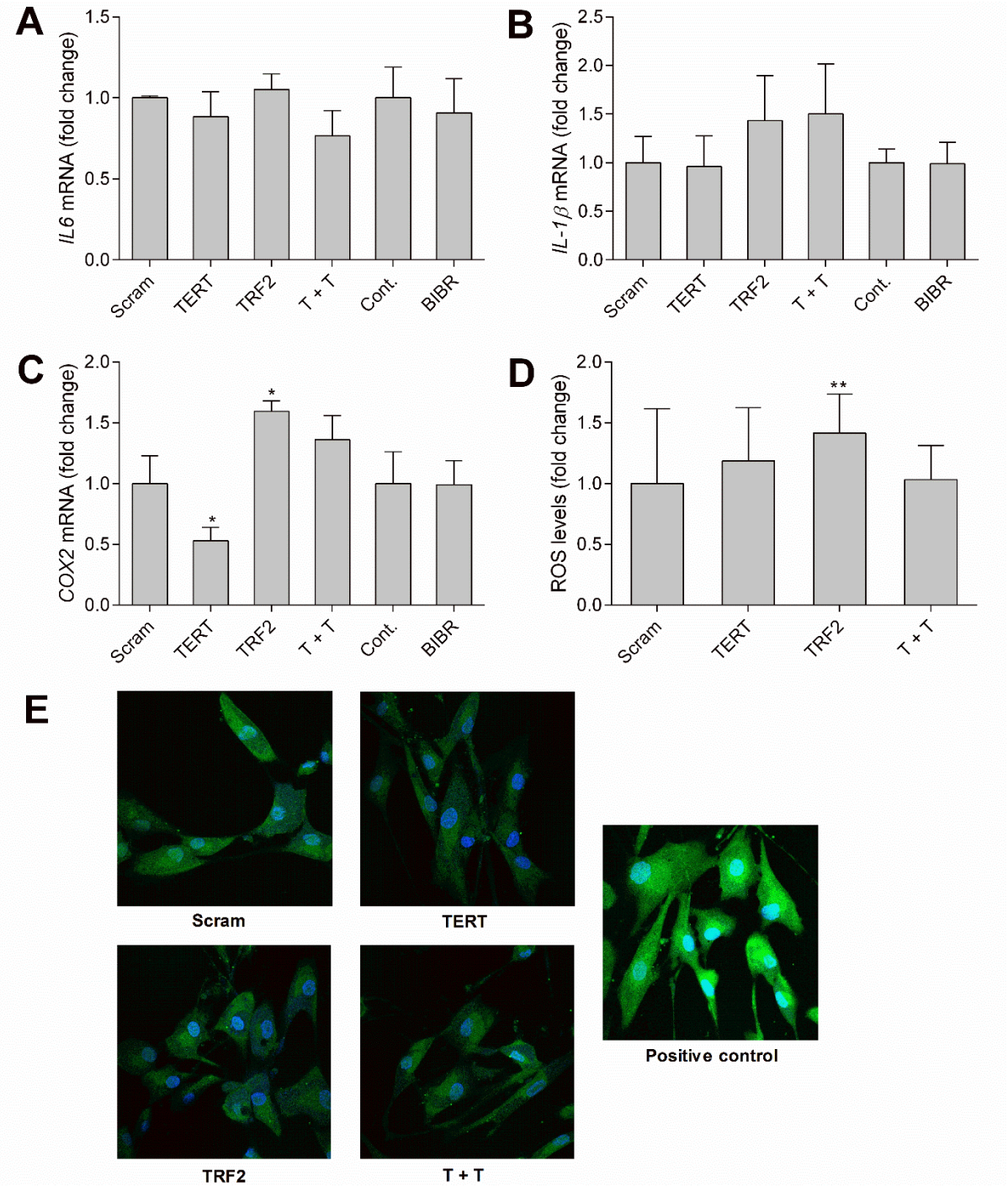

Figure 6. Inflammation and oxidative stress. (A) Inhibiting telomere maintenance did not affect *IL6* or (B) *IL-1β* mRNA levels; (C) *TERT* knockdown decreased and *TRF2* knockdown increased COX2 expression; (D) *TRF2* knockdown increased ROS levels; (E) 6-carboxy-2',7'-dichlorodihydrofluorescein diacetate immunofluorescence for ROS. All HPCs. Data are presented as mean ± SD (n=3 for gene expression and n=25 for ROS) with Scram and Cont. normalised to 1. * indicates $P<0.05$ compared to Scram or Cont. ** indicates $P<0.01$ compared to Scram or Cont. Scram, Scramble siRNA; TERT, siRNA-TERT; TRF2, siRNA-TRF2; T + T, siRNA-TERT and siRNA-TRF2; Cont., no treatment control; BIBR, BIBR1532. *IL6*, Interleukin 6; *IL-1β*, Interleukin-1β; *COX2*, cyclooxygenase-2; ROS, reactive oxygen species.

Proliferation and apoptosis

All treatments significantly reduced the expression of *Mi67* ($P<0.05$) (Figure 7A), the gene for Ki67, which is also a marker of cardiomyocyte proliferation (50). As such, immunocytochemistry and confocal microscopy (Figure 7B) was performed on siRNA-treated cells and revealed that Ki67 was also decreased in these groups (all $P<0.05$) (Figure 7C). Levels of Ki67 were not assessed following treatment with BIBR1532.

To explore if apoptosis accompanied this decrease in proliferation, cleaved PARP western blotting was performed (74) but was only detected in the positive control cells (Figure 7D). In siRNA-treated cells, cleaved caspase 3 was also observed using immunocytochemistry and confocal microscopy (Figure 7E), and a significant increase was detected as a result of *TRF2* knockdown both independently ($P=0.001$) and in conjunction with the *TERT*-siRNA ($P=0.014$) (Figure 7F).

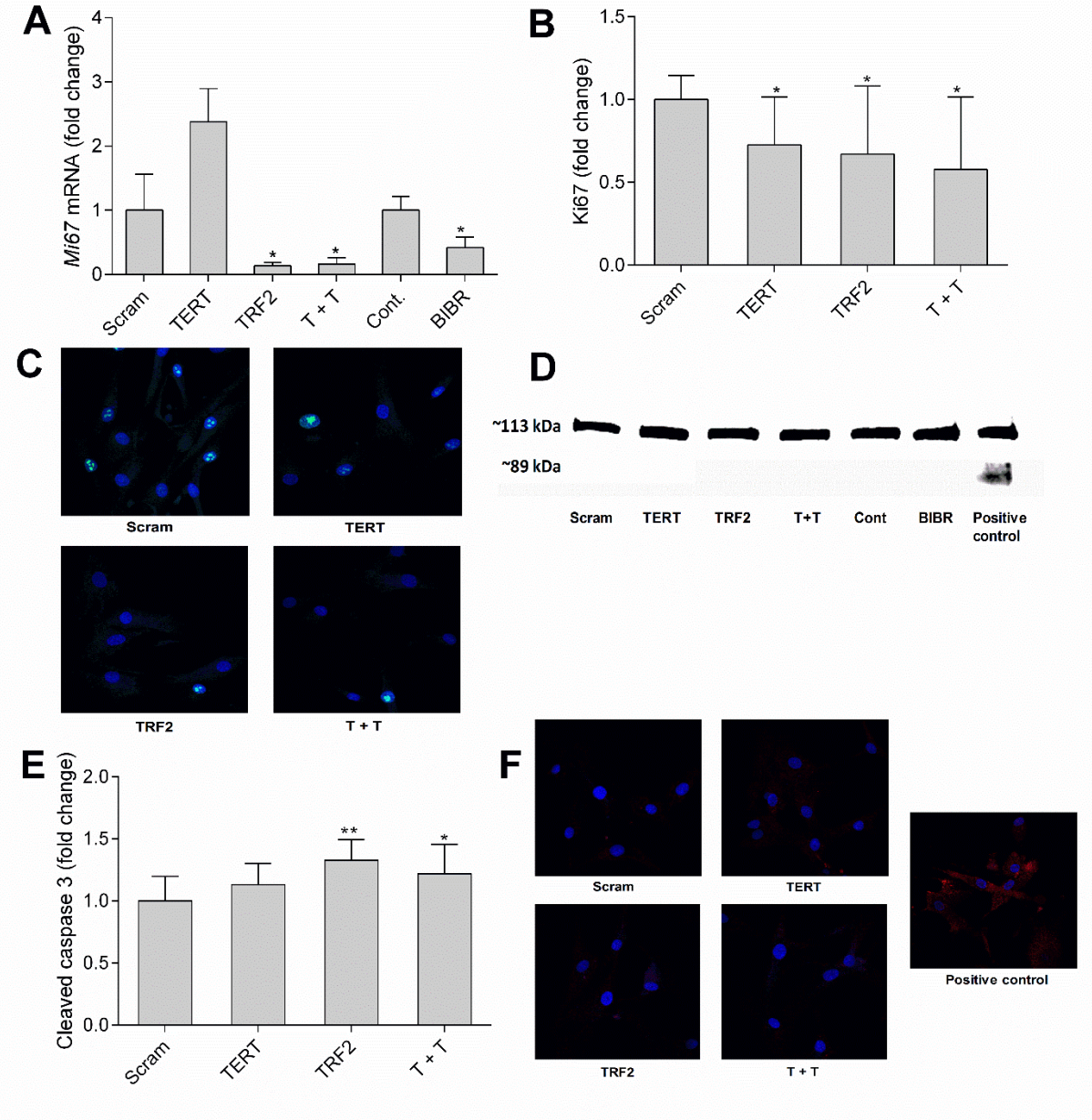

Figure 7. Proliferation and apoptosis. (A) *TRF2* knockdown and BIBR1532 treatment decreased *Mi67* expression; (B) *TERT* and *TRF2* knockdown decreased Ki67 levels; (C) Anti-Ki67 immunocytochemistry; (D) Western blot for PARP; (E) *TRF2* knockdown increased expression of cleaved caspase 3; (F) Anti-cleaved caspase 3 immunocytochemistry. All HPCs. Data are presented as mean ± SD (n=3 for gene expression and n=7 for Ki67 immunocytochemistry) with Scram and Cont. normalised to 1. * indicates $P<0.05$ compared to Scram or Cont. ** indicates $P<0.01$ compared to Scram or Cont. Scram, Scramble siRNA; TERT, siRNA-TERT; TRF2, siRNA-TRF2; T + T, siRNA-TERT and siRNA-TRF2; Cont., no treatment control; BIBR, BIBR1532. *Mi67*, gene for Ki67.

Discussion

The present study is the first to determine the effects of inhibiting *TERT*, *TRF2*, and telomerase activity on human cardiomyocyte hypertrophy. Results demonstrate that each of these methods of inhibiting telomere maintenance is sufficient to increase HPC size, independently of changes in telomere length. In addition, all treatments produced a decrease in HPC proliferation and expression of *MAPK1*, a gene associated with adaptive or 'physiological' cardiac growth. Furthermore, specific *TRF2* knockdown in HPCs was shown to induce oxidative stress, inflammation, apoptosis, and re-expression of the fetal genes and markers of pathological cardiac growth *NPPA* and *NPPB*. These findings indicate that, in addition to the accelerated biological aging and cell loss, lack of telomere maintenance can also induce cardiomyocyte hypertrophy and other molecular and cellular features, such as decreased proliferation, that are characteristic of heart disease.

Although there is little evidence to causally link telomere insufficiency and cardiac hypertrophy, it has been suggested that short telomeres and not defective telomerase determines disease phenotypes (90). To support this suggestion, telomerase-deficient mice are healthy in early generations (15) and humans with mutations in *TERT* are asymptomatic in childhood and sometimes into adulthood (72). Furthermore, genetic anticipation is paralleled by degenerative telomere shortening in both mice (15) and humans (1) with defective telomerase. Findings of the present study, however, suggest that impaired telomerase can cause cardiomyocyte hypertrophy independently of telomere length. More specifically, the ability of the telomerase inhibitor BIBR1532 to induce hypertrophy before decreasing telomere length indicates that HPC size is more effected by insufficient telomere maintenance than telomere length. Interestingly, telomerase-deficient mice present with cardiac hypertrophy in the second generation, before obvious signs of premature aging and disease occur (51). Similarly, cardiac *Tert* and telomerase expression were downregulated in

the hypertrophic heart rats used in Chapter 2 before the onset of cardiac hypertrophy. Moreover, *TERT* mutations in humans lead to dyskeratosis congenita, a bone marrow failure syndrome, usually by 30 years of age (2), potentially obscuring the identification of early-stage cardiac hypertrophy. Therefore, previous findings do not contradict the results of the present study which suggest that telomerase insufficiency may cause cardiac hypertrophy with the subsequent loss of telomere length later coinciding with heart failure (Figure 8).

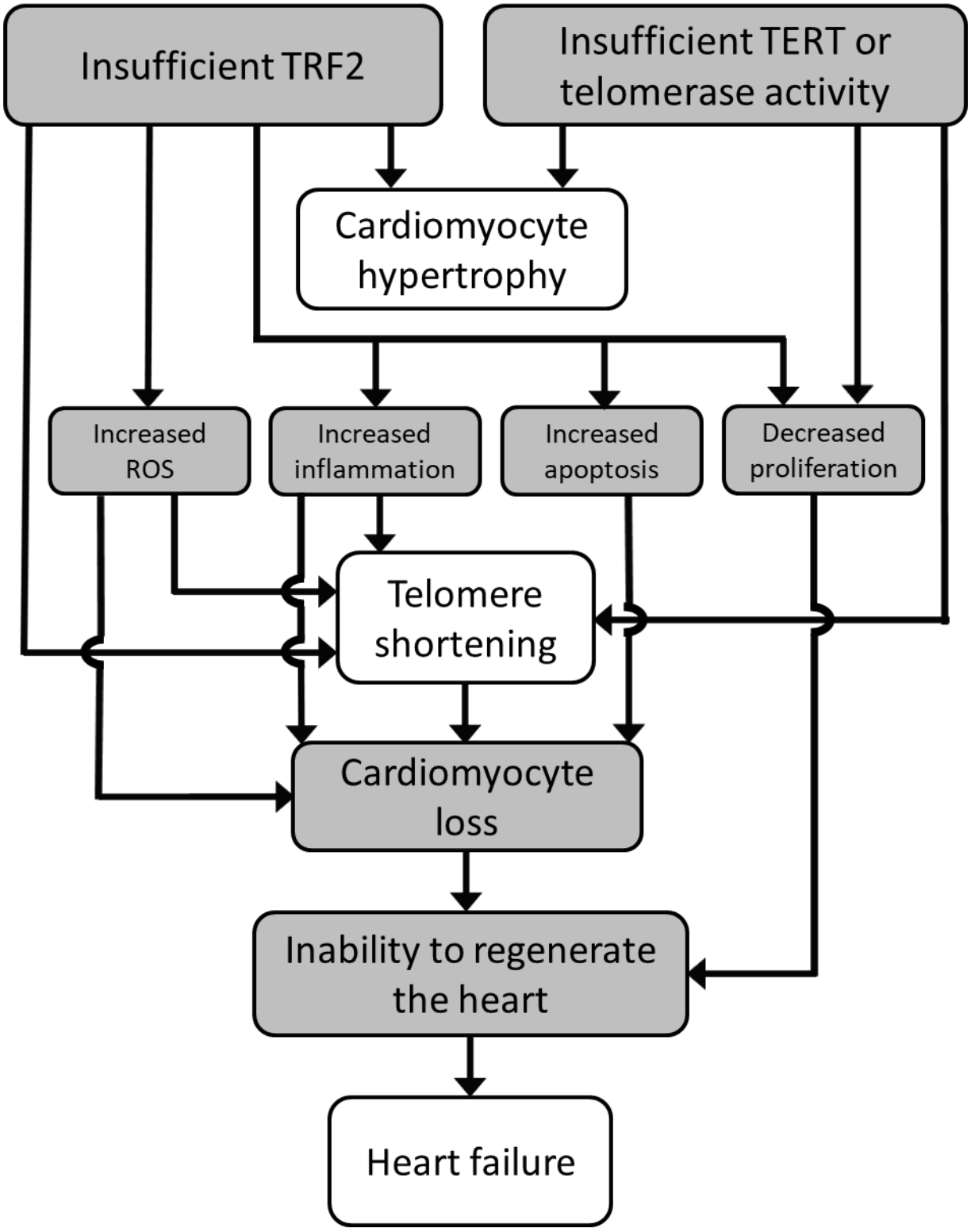

Figure 8. The possible involvement of TERT, TRF2, and telomerase activity in the development of cardiac hypertrophy and heart failure. TERT, telomerase reverse transcriptase; TRF2, telomere repeat-binding factor 2; ROS, reactive oxygen species.

Despite stable telomere length between groups suggesting an important role for telomere maintenance in regulating cardiomyocyte size, only one additional telomere-associated gene was dysregulated as a result of *TERT* or *TRF2* knockdown. The TERT-siRNA, was found to inhibit both *TERT* and its substrate *TERC*. Interestingly, only *TERT* and not *TERC* knockdown inhibits telomerase activity in breast cancer cells (79), although this may not apply to non-cancerous cells (4). Indeed, *Terc* knockout or haploinsufficiency in mice inhibits telomerase activity and predisposes to cardiac hypertrophy to the same extent as $Tert^{-/-}$ and $Tert^{+/-}$ mutants (90). Furthermore, both *Tert* (67) and *Terc* (14) mRNA are rapidly downregulated in the mouse heart in early life, making it difficult to elucidate the effects of these genes on cardiac growth and disease. Despite the similar effects of *Tert* and *Terc* insufficiency, *Terc* mRNA levels are often not assessed following TERT-siRNA knockdown (47, 48, 52, 79), potentially underreporting the significant effects *TERT* suppression on TERC mRNA and its possible role on cell function. In the present study, however, *TRF2* knockdown increased *TERT* expression and, as a result, when TERT- and TRF2-siRNAs were used in conjunction, *TERT* levels were not different than the scramble control. This suggests the stimulatory effects of the TRF2-siRNA on *TERT* expression cancelled out the inhibitory effects of the TERT-siRNA. In contrast to the stable *TERT* expression in the double-knockdown group, *TERC* remained downregulated, suggesting that changes in *TERC* mRNA levels are not a by-product or compensatory mechanism of decreased *TERT* in HPCs. As such, these findings should be interpreted while considering the potential confounding effects of *TERC* knockdown.

In humans and other large, long-lived mammals, *TERT* is located near the telomere, at the end of the chromosome 5. As such, cells with relatively long telomeres can form a chromatin loop with the *TERT* locus and repress *TERT* expression (46). In addition, DNA methylation around the *TERT* region is significantly higher in cells with long telomeres (46).

Furthermore, there are almost 3000 interstitial TTAGGG repeats in the human genome (106) with several around the *TERT* gene (46). These combined mechanisms could explain why the down regulation of *TERT* in early passage cells was detrimental as *TERT* levels may already repressed due to relatively long telomeres. Importantly, TRF2 also interacts with interstitial TTAGGG repeats (76), and siRNA knockdown of *TRF2* has been shown to reduce interactions between telomeres and the *TERT* locus (46). Therefore, the upregulation of *TERT* may be an inevitable consequence of TRF2 insufficiency. Interesting, *Trf2* expression is increased in cardiac-specific *Tert* transgenic mice (67) (the opposite relationship observed in humans) and rodents lack the telomeric location (41) and interstitial TTAGGG repeats around the Tert locus (46). This suggests that TRF2-mediated chromatin looping is an important mechanism of *TERT* regulation in human cells. Like potential off-target effects of *TERT* knockdown, studies have not reported Tert or Terc mRNA or protein levels following *TRF2* knockdown (17, 46, 68, 70) so similar changes may have occurred previously. Indeed, given the well-documented increase in *TERT* and telomerase at the onset of heart disease (50, 82, 97), the pathogenic effects of *TRF2* knockdown may cause a compensatory increase in *TERT* expression.

As the possibility of telomere-independent effects of, and potential interactions between, TERT and TRF2 were anticipated, the telomerase inhibitor BIBR1532 was used to and assess the effects of telomere insufficiency without genetic interference. Although telomerase inhibition did not change *TERT* or *TRF2* expression, it did cause a significant decrease in *TERC*, *TRF1* and *TINF2* mRNA levels. Despite an early report suggesting small molecule telomerase inhibition did not interfere with chromosomal structure (23), BIBR1532 can decrease *TERT* mRNA (28, 47) and TRF2 protein (37) levels, as well increase *TRF1* expression (in contrast to the downregulation of *TRF1* observed in the present study) (28). In this regard, TRF1 helps maintain cell viability after BIBR1532 treatment (104), highlighting

the importance of TRF1, and its interacting factor TINF2, when telomerase activity is impaired. All these changes, however, could be due to cell types, dose, or, in some cases, treatment duration. Nonetheless, BIBR1532 treatment in HPCs, along with the *TERT* and *TRF2* knockdown findings, demonstrate that insufficient telomerase activity, *TERT* and *TERC*, or *TRF2* can induce hypertrophy. Furthermore, as *TERT* expression was unaltered by BIBR1532, *TRF2* was unchanged by the TERT-siRNA, and *TERC* was not affected by *TRF2* knockdown, changes in HPC size can also occur independently of alterations in these genes. Interestingly, however, there was at least one downregulated telomeric gene in the presence of hypertrophy, suggesting that telomere integrity in general influences cardiomyocyte size. This could be due to the complex and precisely regulated interactions that are required for successful telomere maintenance (88).

Changes in chromatin structure can also regulate non-telomeric gene expression in muscle cells (76). Indeed, Robin and colleagues found three genes that changed with decreases in telomere length and one of these, interferon-stimulated gene 15kd, was on chromosome 1p36.22 (76). Interestingly, *NPPA* and *NPPB* also occupy this region but their expression in relation to telomere length and structure has not been determined. In this regard, the decrease in *TRF2* may have prevented the interaction of telomeres with these genes, explaining, at least in part, the upregulation of *NPPA* and *NPPB* following *TRF2* knockdown. Another factor to consider in the context of these findings is that the normal suppression of the fetal gene program coincides with the sharp reduction in *Tert*, *Terc* and telomerase in the perinatal period (19, 96). Although telomere length was unchanged following 48 hours of inhibiting telomere maintenance, it is reasonable to assume that the HPCs used in the present study have shorter telomeres than fetal cardiomyocytes (94, 96). Moreover, if chromosome looping can interact with *NPPA* and *NPPB* then these genes may be more susceptible to re-expression in cells with defective TRF2 or short telomeres.

Therefore, the transcription of *NPPA* and *NPPB* in the presence of short telomeres and other epigenetic changes associated with aging could explain why these genes are maladaptive upon adult re-expression.

In addition to the upregulation of fetal genes, there was also a decrease in *MAPK1* expression, a gene associated with adaptive cardiac growth (21), in all treatment groups. Importantly, MAPK signalling pathways can protect cardiomyocytes (32, 83) and other cells (71) from apoptosis *in vitro*. As a decrease in *MAPK1* mRNA levels was consistent across all treatment groups, this suggests there may be a link between telomere maintenance and *MAPK1* in HPCs. Interestingly, estrogen-induced transcription of telomerase is mediated through MAPK signalling pathways (111). In addition, *MAPK1* expression is associated with the differential expression of microRNAs determined by TERT genotype in colon cancer (86). Furthermore, *MAPK1* was upregulated in agonist-treated groups in the previous Chapter, along with increased TERC mRNA levels. Taken together, these findings suggest that telomere maintenance could influence MAPK signalling and hence whether cardiac growth is adaptive or harmful.

Another consistent feature of cardiomyocyte hypertrophy observed in this Chapter was a decrease in HPC proliferation. Similarly, previous research has shown that cardiomyocyte size is inversely correlated with proliferative ability and telomere length (78) – both of which can be caused by TERT-siRNA (52), TRF2-siRNA (70), or BIBR1532 (47) treatment. Furthermore, the decrease in proliferation shortly after birth is believed to be the mechanism responsible for restricting the majority of cardiac growth throughout life to hypertrophy (92). In this regard, adult cardiomyocytes may retain this signalling mechanism which would explain the increase in cell size detected in these treatment groups. In addition to decreasing proliferation, inhibiting *TERT* or telomerase seems to have an overall inhibitory effect on cells. Indeed, there were no stimulatory effects on any of the genes measured as a

result of BIBR1532 treatment, with similar findings in the TERT-siRNA group. In previous studies, siRNA inhibition of *TERT* (34) and treatment with BIBR1532 (84) has been shown to increase the cardiotoxicity of doxorubicin as well as other chemotherapy agents (62, 100). Similarly, wild type mice, if supplied with running wheels, are immune from the apoptotic effects of doxorubicin but *Terc*$^{-/-}$ mice are not (185). Despite the decrease in proliferation and expression of some protective genes, however, there were no signs of apoptosis as a result of *TERT* knockdown or telomerase inhibition with BIBR1532 in HPCs. Importantly, like the telomere shortening observed following ten days of BIBR1532 treatment, apoptosis is also an inevitable consequence of insufficient telomere maintenance (13, 16) and hence would occur with longer treatments.

Unlike the acute knockdown of *TERT* or suppression of telomerase activity, the TRF2-siRNA induced apoptosis in HPCs after only 48 hours of treatment. This is consistent with the effects of *TRF2* knockdown in renal carcinoma cells (70) and could be considered a result of chromosomal instability that accompanies TRF2 insufficiency and not necessarily short telomere length (57). For instance, enhanced TRF2 protein levels but not alterations in telomere length are associated with endothelial cell viability (89). In addition, long telomeres lacking TRF2 can still signal apoptosis, probably due to resembling damaged DNA (56) and it has been suggested that this may occur in cardiomyocytes (68). Interestingly, oxidative stress and inflammation, two established causes of cardiomyocyte apoptosis (60, 99), were both observed in the TRF2-siRNA group only. In this regard, TRF2 protein levels increase (and telomeres shorten) in response to oxidative stress in mouse skeletal muscle (53), suggesting that cells lacking TRF2 are less equipped to counteract normal levels of endogenous oxidative stress. Similarly, loss of TRF2 induces inflammation in arteries (64) which can lead to DNA damage and senescence associated inflammatory cytokine secretion (77) and Trf2 deficiency in mouse embryos causes inflammation and apoptosis (57). These

findings highlight the ability of short-term *TRF2* knockdown in HPCs to simulate many of the molecular and cellular characteristics of pathological cardiac hypertrophy and hence its potential utility as a model of human cardiac disease.

The present study also had some limitations which could be addressed with future research. Firstly, more time points could be used to assess possible transient changes in gene expression (24) and if other factors, such as decreased proliferation, precede increases in cell size. Secondly, telomere length measurements using quantitative fluorescent *in situ* hybridisation or a similar technique could further elucidate the role of telomeres in the development of cardiac hypertrophy by providing more sensitive and cell-specific measurements of telomere length (37). This could also be used to correlate telomere length and proliferation with cardiomyocyte size in individual cells (78). Further to this, markers of cytokinesis could be used to more accurately measure proliferation as Ki67 is also associated with changes in DNA content that may not result in cell division (98). In addition, the potential ability of TRF2, telomerase activity, and telomere length to regulate cell-specific genes is an important area for future research. Indeed, it would be useful to determine the mechanism through which *TRF2* knockdown induced *NPPA* and *NPPB* expression. It also needs to be noted that all cells used in this study came from one individual and are not true primary cardiomyocytes but rather a population of replicating cardiac progenitor cells. As such, these findings need to be validated in other models.

In conclusion, the findings of the present study demonstrate that disruptions in telomere maintenance are sufficient to induce cardiomyocyte hypertrophy independently of changes telomere length. Given the presence of short telomeres and cardiac hypertrophy in diabetes, smoking, alcoholism, chemotherapy treatment, and other risk factors for heart failure (18), inhibiting telomere maintenance could be a useful model of cardiac hypertrophy and early stage heart disease. TERT-siRNA and BIBR1532 may have contributed to HPC

hypertrophy through decreasing proliferation whereas TRF2-siRNA could have additional mechanisms such as increased *NPPA* and *NPPB*, oxidative stress, and apoptosis. The role of TRF2 in maintaining telomere loops suggests an important function for telomere structure in regulating cardiomyocyte size and hypertrophic gene expression. This study provides new evidence and elucidates the role telomeres can play in the development of cardiac hypertrophy and hence heart failure.

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Chapter 6

General Discussion and Conclusions

Recap and summary

As discussed in Chapter 1, there are currently no effective strategies to prevent the degenerative cardiomyocyte loss that leads to heart failure (34, 82). Association studies have shown that short telomere length (TL) is a molecular feature of heart failure (14, 53) but the contribution of telomere insufficiency in the pathogenesis of this disease is unknown. In this regard, cardiac hypertrophy precedes heart failure (35, 54) and is also associated with changes in cardiomyocyte TL (52, 63) and maintenance (65, 73). Therefore, telomeric aberrations in cardiac hypertrophy could explain the cell loss that is characteristic of subsequent heart failure as well as identify therapeutic targets and guide preventative interventions. As such, the overall aim of this thesis was to determine the role of telomeres in the development of cardiac hypertrophy using a combination of *in vivo* and *in vitro* models.

To formally test the possibility of a causal role for telomeres in the development of cardiac hypertrophy, it was hypothesised that decreases in TL and/or maintenance would increase cardiomyocyte size. In Chapters 2 and 3, polygenic and environmental rat models of cardiac hypertrophy were used, respectively. In Chapters 4 and 5, human primary cardiomyocytes (HPCs) were grown *in vitro* and treated with hypertrophic agonists or inhibitors of telomere maintenance. Although these research designs differ, *Tert*, *Terc*, and *Ppp1r10* (also known, and hereafter referred to, as *Pnuts*) mRNA levels, TL, as well as measures of cardiac or cardiomyocyte hypertrophy were determined in all these Chapters. These results are summarised in Figure 1 and then discussed in more detail below, along with other key findings from this Thesis.

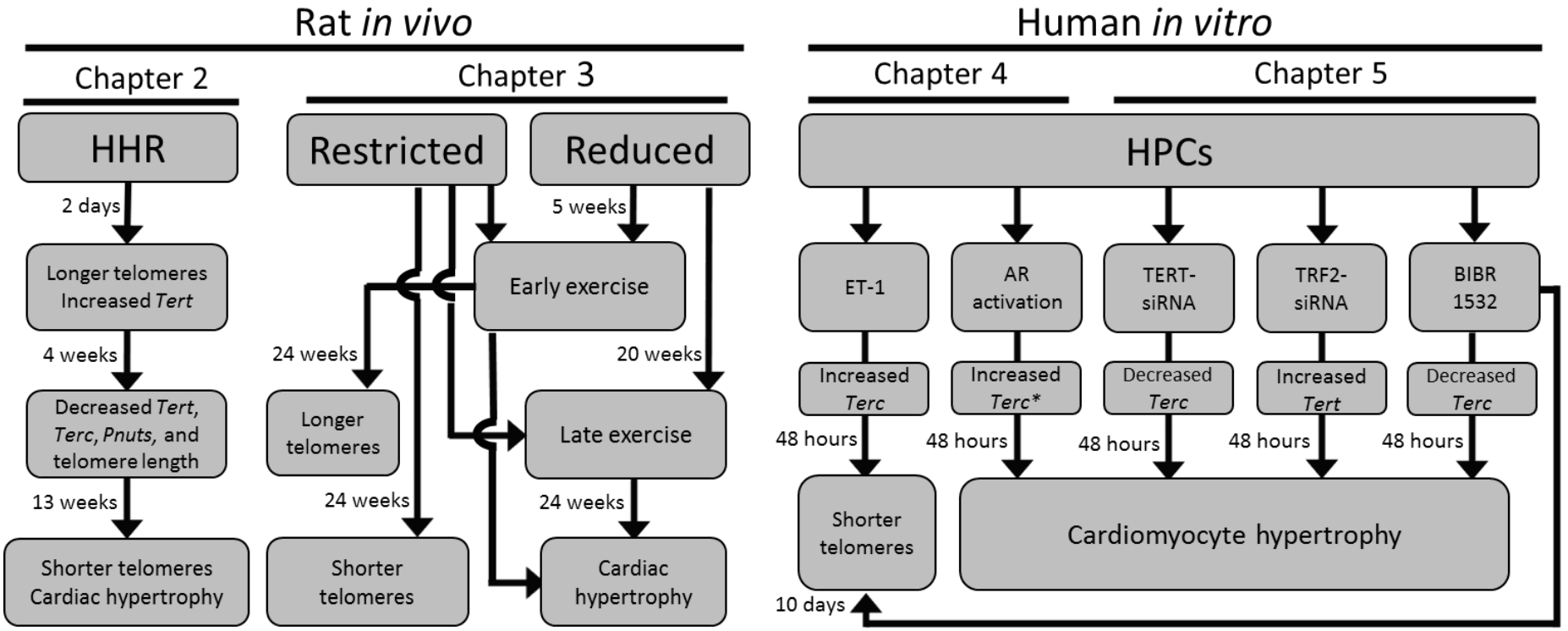

Figure 1. A summary of comparable findings between Chapters. *Tert* was upregulated at 2 days but reduced at 4 weeks of age along with telomere length, *Terc*, and *Pnuts*. Reduced rats had increased *Tert* expression relative to Control rats, *Pnuts* was increased in Restricted rats but there were no changes in *Terc* mRNA levels in either group (not shown). Adrenergic and endothelin receptor activation in HPCs increased *TERC* mRNA levels, which paralleled increases in cell size, but had no effect on *TERT* or *PNUTS* expression. Inhibiting telomere maintenance in HPCs involved changes in *TERT* and *TERC* but not *PNUTS* expression although hypertrophy also occurred without the involvement of these genes. Telomere length in the HHR was longer at 2 days but shorter by 13 weeks of age. Telomeres were shorter in Restricted rats but longer after early exercise treatment in all groups. ET-1 reduced cardiomyocyte telomere length after 48 hours, 10 days, and 16 days of treatment. BIBR1532 shortened HPC telomeres after 10 days. Cardiac hypertrophy occurred in the HHR by 13 weeks of age. Unlike previous cohorts, Restricted and Reduced rats did not present with cardiac hypertrophy, although exercise increased cardiac weight index in all groups. Endothelin and adrenergic receptor activation, TERT-siRNA and TRF2-siRNA knockdown, and inhibiting telomerase with BIBR1532 induced HPC hypertrophy. *with the exception of adrenaline treatment. AR, adrenergic receptor; BIBR1532, 2-((E)-3-naphthalen-2-yl-but-2-enoylamino)-benzoic acid; ET-1, endothelin-1; HHR, hypertrophic heart rat; HPCs, human primary cardiomyocytes; *Pnuts*, protein phosphatase 1 regulatory subunit 10.

Cardiac telomeres in rat models of human disease

In Chapter 2, cardiac and cardiomyocyte TL was determined in the hypertrophic heart rat (HHR) before, during, and after the development of cardiac hypertrophy. This work revealed vastly different patterns of telomere attrition and telomeric gene regulation between the HHR and its control strain. In the HHR, cardiac TL was longer at birth and 38 weeks of age but shorter at 13 weeks of age (the onset of cardiac hypertrophy). Somewhat surprisingly, there was no difference in cardiac TL at 52 weeks of age, around the time the HHR suffers from heart failure (57). Importantly, telomere shortening paralleled increases in cardiac weight index (CWI) in both strains, providing some evidence in support of the hypothesis that telomere attrition increases cardiac mass. As both cardiac growth (25, 50) and telomere shortening (9, 42) are normal features of aging, however, these findings do not preclude a consequential or confounding role for telomeres in the development of cardiac hypertrophy.

One limitation of many studies on telomeres in heart disease is the use of circulating leukocyte TL as it may not reflect cardiac TL (23) or contribute to the pathogenesis of heart failure (64). To address this, Chapter 2 determined TL in circulating leukocytes at the onset of cardiac hypertrophy and in the presence of shorter cardiac TL (13 weeks of age). In accordance with the heart, leukocyte TL was shorter in the HHR and there was significant correlation between these two variables. In the NHR, however, leukocyte TL was not significantly correlated with cardiac TL, demonstrating that leukocyte telomere length is not always representative of the heart. Indeed, previous studies have reported significantly longer leukocyte telomeres in the presence of cardiac hypertrophy (37, 76) in contrast to the short leukocyte telomeres found in heart failure (75). While this could be due, at least in part, to physiological cardiac hypertrophy, which is associated with longer telomeres (46), or the upregulation of TERT that has been found in early-stage heart failure (65, 73), it is also possible that TL was shorter in hypertrophic hearts and that this was not reflected in

leukocytes. Furthermore, if short telomeres determine disease phenotypes as suggested (69), then leukocyte TL is unlikely to be a useful biomarker as it would not be detectable in the early and reversible stages of disease. In this regard, a telomeric variable such as *Tert* (discussed below) could be a more useful target for disease identification and prevention.

Following on from the investigation into polygenic cardiac hypertrophy, the aim of Chapter 3 was to determine if changes in cardiac telomeres were present in an environmental model of cardiac hypertrophy caused by growth restriction in early life. Unlike previous research using Restricted and Reduced rats (78), cardiac hypertrophy was not present at 24 weeks of age in the cohorts used in this Chapter. While this prevented the testing of the main hypothesis of this Thesis, it did not preclude the investigation of growth-restriction and exercise effects on cardiac telomeres. Indeed, a major finding of Chapter 3 was that Restricted rats had shorter cardiac telomeres than Reduced and Control rats in all exercise groups. Furthermore, cardiac telomere length was positively correlated with body weight and heart weight but not CWI. Interestingly, the HHR is also born small and remains lighter throughout life, suggesting low bodyweight could influence cardiac TL. If longer cardiac telomeres were detected in other low birthweight individuals this would favour the suggestion put forth in Chapter 2 that fewer cardiomyocyte divisions in early life delays cardiac growth, prevents proper maturation of the heart, and predisposes to cardiac hypertrophy in later life. This would be interesting to determine as long cardiac telomeres at birth may be a genetic trait of the HHR that is unrelated to the development of cardiac hypertrophy.

Significant evidence is accumulating that highlights the effects of adverse events in early life on adult TL and disease risk. For instance, recent meta-analyses have demonstrated that stress (18), separation (40), abuse, neglect, and other traumas (62) in childhood are associated with short leukocyte telomere length in adulthood. In addition to expanding this phenomenon to telomeres in the heart, Chapter 3 demonstrated that exercise training in

adolescence can attenuate the cardiac telomere shortening caused by fetal growth restriction. Although the importance of genetic predisposition or early environmental effects on later cardiac disease is becoming obvious, the relevance of animal models needs to be continually validated to ensure studies are representative of human disease. The absence of cardiac hypertrophy in Restricted and Reduced rats demonstrates that animal models do not always present with the same characteristics across cohorts. Nonetheless, early exercise training attenuated cardiac telomere attrition even in normal birth weight Reduced and Control rats, highlighting the potential of exercise treatment in the prevention of heart failure.

Levels of the proapoptotic and hypertrophic miR-34a were assessed in Chapter 2 and found to be significantly overexpressed in the HHR in early life only. As such, miR-34a expression was not determined in the 24-week old Restricted and Reduced rats used in Chapter 3. However, *Trf2*, *Pnuts*, and *Sirt1* were all positively correlated with one another, suggesting that they may be controlled by a master regulatory agent such as miR34a. In support of this suggestion, these genes are all direct or indirect targets of miR-34a (10, 80). In Chapter 2, however, *Pnuts* was only weakly correlated with miR-34a expression at any of the ages and neither *Pnuts* or *Sirt1* were found to reflect changes in telomere length or cardiomyocyte/cardiac size in any of the Studies in this Thesis. An important factor to consider, however, is that telomeric genes (13) and miR-34a expression (21) are transiently upregulated shortly after exercise and thus may not be dysregulated under normal conditions. With this in mind, the anti-hypertrophic and cardio-protective effects of miR-34a inhibition in mice (10, 21) may also maintain cardiac function in humans.

Telomeres in human and rat *in vitro* models of cardiac hypertrophy

Given the recent evidence of differences in telomere regulation between species (36) and the absence of cardiac hypertrophy in Restricted and Reduced rats, Chapter 4 of this Thesis focused on adult human primary cardiomyocytes (HPCs) *in vitro*. The common rat embryonic cardiomyoblast (H9c2) cell line was also used for comparison. Both HPCs and H9c2 cells were treated with the hypertrophic agonists adrenaline (Adren), angiotensin-II (Ang II), endothelin-1 (ET-1), isoproterenol (ISO), and phenylephrine (PE) to induce hypertrophy. All five agonists significantly increased HPC and H9c2 cell size, however only two out of eight selected genes that showed patterns of differential expression in the HPCs were detected in H9c2 cells (*IL-6* and *Mi67*, markers of inflammation and proliferation, respectively). This further demonstrates that key differences exist between primary and immortalised, human and rat, and/or adult and embryonic cell types. Interestingly, in both HPCs and H9c2 cells, there were no differences in *TERT* mRNA levels and, with the exception of ISO-treated HPCs, very few alterations in the genes encoding the protective (shelterin) proteins. This demonstrates that changes in telomere length or telomeric gene expression are not required for cardiomyocyte hypertrophy. Another key finding of Chapter 4 was the reduction in TL after only 48 hours of ET-1 treatment. Follow up research confirmed that ET-1 shortened HPC telomeres in a time dependent manner, highlighting a novel pathophysiological role in the heart that is independent of the vasoconstrictive and hypertensive effects of this agonist.

In Chapter 5, HPCs were grown *in vitro* and treated with TERT- and TRF2-siRNAs and the small molecule telomerase inhibitor BIBR1532 to determine if aberrations in telomere maintenance could induce hypertrophy (the inverse design of Chapter 4). Interestingly, all three of these treatments significantly increased HPC size, demonstrating that inhibiting telomere maintenance is sufficient to cause cardiomyocyte growth,

independently of changes in TL. Although cell-specific and non-telomeric functions have been reported for TERT, telomerase (15), and TRF2 (36), this is the first research to demonstrate that these telomeric regulators may have hypertrophic roles in human cardiomyocytes. Consistent with the stable TLs in hypertrophic HPCs, Terai and colleagues found only a 3bp decrease in TL with each one gram increase in heart weight (70). In this regard, if cardiac hypertrophy is viewed as an asymptomatic and reversible condition in its early stages (24, 25), then the suggestion that telomere shortening determines disease is not contradicted by these findings. Indeed, telomere shortening is a necessary consequence of insufficient telomere maintenance (2, 8). Furthermore, a causal role for telomerase and/or TRF2 in the development of cardiac hypertrophy could potentially be used to identify and treat cardiac hypertrophy before the onset of heart failure and telomere shortening. As the preservation of telomeres is a necessary step in oncogenic transformation (9), such treatments would have to be targeted specifically to cardiomyocytes to avoid an increased risk of cancer.

Telomeric regulators as hypertrophic and cardiac risk factors

When the findings of Chapter 2 are considered from the perspective of telomere regulation and not length, the rapid decrease in telomerase activity in the HHR early in life also offers an explanation for the development of cardiac hypertrophy in this model. Similarly, *TERT* knockdown HPC hypertrophy in Chapter 5. Cardiac and cardiomyocyte hypertrophy, however, was also associated with increased *TERT* expression in both the HHR and HPCs. This is consistent with the literature, where studies have found that mice lacking (39) or overexpressing (5, 52) *Tert* develop cardiac hypertrophy. In addition to fluctuations in *TERT*, significant increases and decreases in *TERC* were also detected in hypertrophic hearts or cardiomyocytes in this Thesis. Moreover, *Trf2* expression is increased in cardiac-specific *Tert* transgenic mice (52) which is the opposite of what was observed in the HPCs. Therefore, despite the importance in *TERT*, *TERC*, and *TRF2* in maintaining cardiac size, hypertrophy can occur in the presence of increased, decrease or stable levels of these genes (Table 1). This suggests that there is more than one telomeric pathway that can influence cardiomyocyte size in both rodents and humans. In this regard, these findings also demonstrate that changes in telomeric gene expression are **s**ufficient but not necessary for the development of cardiac hypertrophy in rodents and humans. Although this does not take into account the enzymatic activity of TERT and TRF2, short telomere length is the inevitable consequence of insufficient telomere maintenance, even at the level of gene expression (2, 8). Therefore, this still has important consequences as short telomere length is a universal feature of poor cardiac health (14, 27, 53).

Table 1. Cardiac *TERT*, *TERC*, and *TRF2* mRNA levels in models of cardiac hypertrophy.

Model/treatment	Cardiac *TERT*	Cardiac *TERC*	Cardiac *TRF2*
HHRs*	Unchanged	Unchanged	Not determined
Tert and Terc$^{-/-}$ mice	Absent	Absent	Not determined
Tert and Terc$^{+/-}$ mice	Decreased	Decreased	Not determined
Tert transgenic mice	Increased	Not determined	Increased
Agonist-treated HPCs$^{\#}$	Unchanged	Increased	Unchanged
TERT-siRNA HPCs$^{\#}$	Decreased	Decreased	Unchanged
TRF2-siRNA HPCs$^{\#}$	Increased	Unchanged	Decreased
TERT- and TRF2-siRNA HPCs$^{\#}$	Unchanged	Decreased	Decreased
BIBR1532 HPCs$^{\#}$	Unchanged	Decreased	Unchanged

*at the onset of hypertrophy (13-weeks of age). $^{\#}$after 48 hours of treatment. HHRs, hypertrophic heart rats; HPCs, human primary cardiomyocytes; Tert, telomerase reverse transcriptase; Terc, telomerase RNA component; Trf2, telomere repeat-binding factor 2; BIBR1532, 2-((E)-3-naphthalen-2-yl-but-2-enoylamino)-benzoic acid.

Like telomeric gene expression, significant increases as well as decreases in cardiomyocyte proliferation were detected in the presence of hypertrophy. It should be noted that proliferation was measured using Ki67 antigen expression (as performed previously (7, 43)), which is also associated with changes in cardiomyocyte ploidy and not necessarily completed cell division (74). Interestingly, however, there were clear trends within groups with all agonist treatments increasing proliferation (Chapter 4) and all telomere-inhibiting treatments decreasing proliferation (Chapter 5). As cardiomyocyte proliferation is the driving feature of cardiac repair (58, 59), inhibiting telomere maintenance may better reflect the phenotype of pathological cardiac hypertrophy that leads to degenerative heart failure. Furthermore, inhibiting telomere maintenance, particularly with *TRF2* knockdown, caused a much greater increase in HPC size than any of the agonists (~3 compared to ~1.5-fold, respectively). As only small cardiomyocytes are capable of undergoing replication in both human and animals (14, 39, 72), greater increases in cell size may lead to decreased proliferation. Moreover, as increased proliferation decreases human cardiomyocyte TL (6) agonist stimulation could cause telomeres to shorten through repeated cell divisions. This is one possible explanation of the early events leading to the telomere attrition and replicative senescence characteristic of heart failure (14, 53, 63, 71). In this regard, Restricted rats (Chapter3) have upregulated expression of the angiotensin-II type 1 receptor (AT_1) (78) which may also be involved in the pathophysiology of cardiac telomere attrition following growth restriction. Similarly, the decrease in telomere length, *Tert*, and *Pnuts* expression observed in young HHRs (Chapter 2) may reduce cardiomyocyte proliferative ability and predispose to cardiac hypertrophy.

Corresponding to changes in proliferation, mRNA levels of the cardioprotective gene mitogen activated protein kinase 1 (*MAPK1*) were also higher in all agonist-treated HPCs (Chapter 4) and downregulated after inhibiting telomere maintenance (Chapter 5).

Interestingly, research suggests *MAPK1* may also be involved in telomere maintenance. For instance, estrogen can stimulate telomerase activity through mitogen activated protein kinase (MAPK) pathways (84) and *MAPK1* expression in colon cancer is associated with TERT genotype (67). Similarly, decreased *Mapk1* expression from siRNA knockdown decreased proliferation in H9c2 cells (83), potentially predisposing to hypertrophy. In regard to heart function, *Mapk1* overexpression in mice leads to cardiac hypertrophy while haploinsufficiency or knockout of *Mapk1* decreases cardiac performance (60). As with telomerase insufficiency in mice, cardiac-specific inhibition of *Mapk1* is required to rule out potential confounding effects of systemic gene deletion. In contrast to some telomeric proteins, evidence suggests that the function of MAPK1 is evolutionarily conserved. For instance, specific Thr188 phosphorylation of MAPK1 has been detected in hypertrophic and failing human hearts (44) and mutations at these sites in mice prevented the development of cardiac hypertrophy (44). Findings from the HPCs in Chapters 4 and 5 also suggest a dynamic role for *MAPK1* in the development of human cardiac hypertrophy but the mechanisms involved are beyond the scope of this Thesis. Nonetheless, the decrease in *MAPK1* expression, in addition to the pronounced hypertrophy and decreased proliferation, further suggests that inhibiting telomere maintenance better reflects the molecular phenotype of pathological cardiac hypertrophy than agonist stimulation (Figure 2).

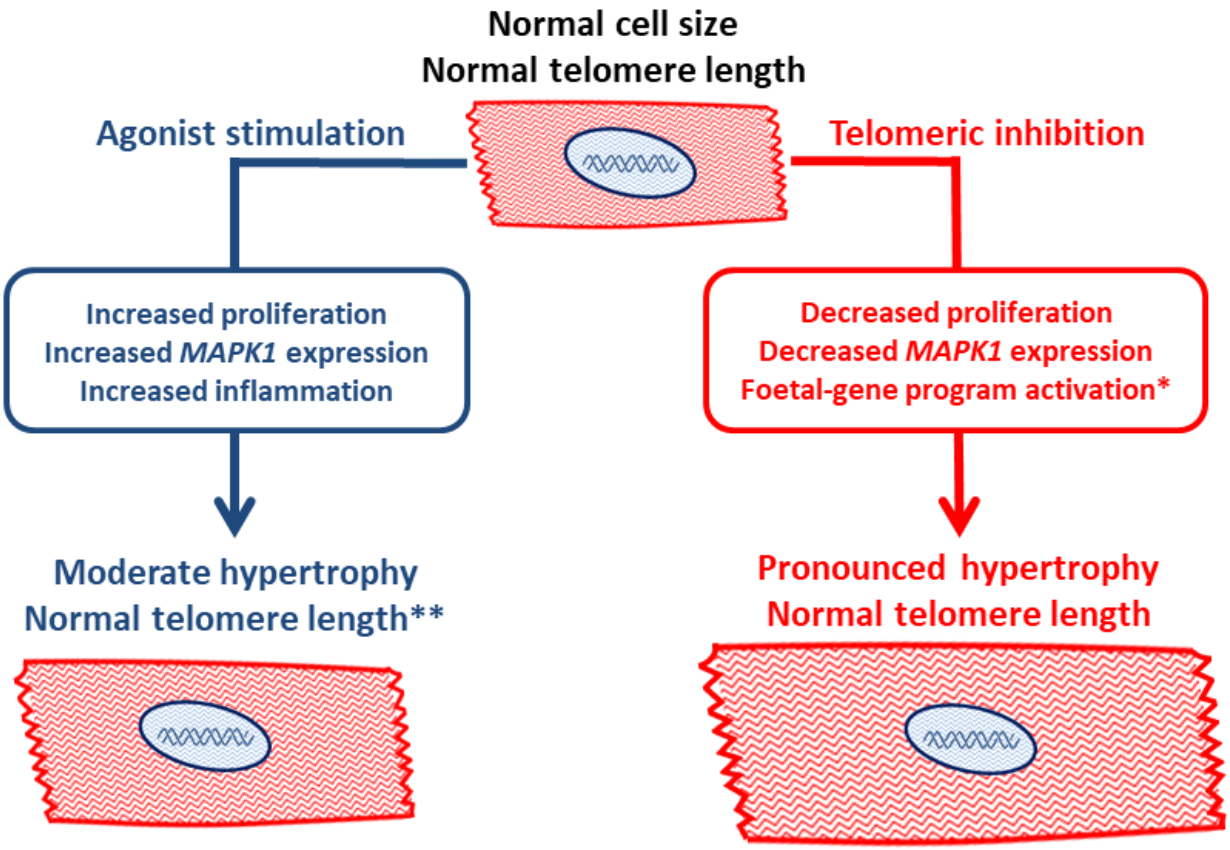

Figure 2. Telomere inhibition induces a more pathological phenotype overall than agonist stimulation. **TRF2* knockdown only. **with the exception of ET-1.

Another key finding of Chapter 5 was the increase in the fetal genes and markers of heart failure *NPPA* and *NPPB* caused by *TRF2* knockdown. This could be due to a decrease in chromatin looping with 1p36.22 locus where these genes are located. For instance, cells with long telomeres can regulate the expression of genes positioned near the ends of chromosomes, including the 1p36.22 locus (36). Importantly, TRF2 is crucial in facilitating these interactions by maintaining telomere structure and interacting with TTAGGG repeats throughout the genome (36). Although no changes in *NPPA* or *NPPB* were detected after telomerase inhibition in HPCs, previous research has indicated that telomerase may also be able to regulate hypertrophic gene expression. For instance, endothelin-converting enzyme (ECE-1) mRNA expression and circulating ET-1 levels are higher in telomerase-deficient mice, which is considered the cause of hypertension in these animals (55). Interestingly, significant decreases in TL occur only after the development of hypertension in spontaneously hypertensive mice (16), further suggesting a role for telomere maintenance and not length in disease onset. Taken together these findings demonstrate that telomeric factors can regulate hypertrophic genes and that loss of telomere function could directly influence the pathogenesis of cardiac hypertrophy.

Although fetal genes are still considered key biomarkers of pathological cardiac hypertrophy and heart failure (17), inflammatory markers were the most dysregulated genes detected in explanted human hearts (12). Indeed, interleukin-6 (*IL6*) and interleukin-1β (*IL-1β*) were upregulated 11.54- and 8.23-fold, respectively while *NPPA* or *NPPB* were not listed as having a fold change >1.8. Other studies have also demonstrated that *NPPA* and *NPPB* expression in adulthood does not always reflect pathological cardiac hypertrophy (1, 66, 79). In this regard, the agonist-treated cells (Chapter 4) accurately modelled pathological cardiac hypertrophy, causing the upregulation of at least one inflammatory marker in all groups. Furthermore, inflammation can illicit reactive oxygen species (ROS) production (30) a potent

cause of telomere attrition (19) and cardiomyocyte apoptosis (77). In this regard, ROS was only induced by Ang II, ET-1, TRF2-siRNA treatments, all of which caused (or, in the case of Ang-II, are known to cause (20)) profound, age-associated changes in cardiomyocytes. Interestingly, ROS activity is also increased in cultured embryonic *Terc*$^{-/-}$ deficient cells, but treatment with antioxidants can reduce ECE-1 promoter activity, potentially preventing the short-term consequences of telomerase insufficiency (55). Taken together, these findings demonstrate that, in addition to telomere shortening and cardiomyocyte apoptosis, ROS may be linked to hypertrophic gene expression and thereby contribute to the development of cardiac hypertrophy.

Future research

Animal models

There are several questions raised from the findings of this thesis which could be addressed in future studies. Firstly, Trf2 levels, along with other important regulators of TL, such as inflammation (33, 41, 49) and oxidative stress (45, 77), could be determined in HHR and other animal models to elucidate the interactions between hypertrophic and telomeric risk factors. Secondly, determining the efficacy of interventions to reduce the severity of genetic predispositions or environmental insults to disease, is an important area of future research. In this regard, exercise treatments could be performed on the HHR as well as dietary interventions, such as calorie restriction and antioxidant treatment, which have all demonstrated utility in preserving telomeres, heart function, and longevity (29, 47, 48). Thirdly, cardiac telomeres could be measured in young animals predisposed to disease, such as Restricted rats, to determine if they suffer from rapid telomere attrition during early life or *in utero* and if this is involved in the pathogenesis of heart disease. To confirm this, it would also be necessary to house a subset of animals until death and perform post-mortem examinations to ensure the disease being studied is accurately reflected in the model. In this regard, the differences in telomeres and heart function between animals and humans should be carefully considered when designing future studies.

Human *in vitro* models

To elucidate the pathophysiology of human cardiomyocyte growth, more timepoints during the development hypertrophy are required. For instance, the 48-hour treatments used in Chapters 4 and 5 could have missed transient changes in gene expression, particularly genes involved in telomere maintenance (13) and the hypertrophic response (32). Earlier timepoints, such as 6-hours after treatment, would minimise this possibility as well as investigate if the changes in proliferation, inflammation, *TERC* or *MAPK1* expression

reported in Chapters 4 and 5 occur before or after increases in cell size. Similarly, longer treatments lasting several days or weeks could be performed to determine when significant telomere shortening occurs after the development of hypertrophy. In this regard, the increasingly-available CRISPR-Cas9 technology could be used to create useful models of telomere insufficiency and thus cardiac hypertrophy *in vitro*. As ET-1 is produced by cardiomyocytes (81) and upregulated in *Terc*$^{-/-}$ mice (55), ET-1 levels could be determined in human models of telomere insufficiency in search for hypertrophic pathways common to agonist stimulation and telomere maintenance. In contrast, it would also be useful to determine the effects of cardiomyocyte-specific TRF2 overexpression in human cells and if this could rescue or prevent hypertrophy. Results from this Thesis have also provided the foundation and rationale to pursue more detailed analyses on factors implicated in changes to HPC size, such as specific phosphorylation of MAPKs.

Given the inconsistent changes in *TERT*, *TERC*, and *TRF2* expression (Table 1) in the presence of hypertrophy, future studies could search for novel telomeric factors whose expression reliably reflects cell size. One such factor could be Telomere Repeat-Containing RNA (TERRA), a long noncoding RNA transcribed from subtelomeric promoters. In support of this suggestion, TERRA has been shown to directly interact with TRF2 (22), inhibit telomerase (61), facilitate telomere looping, and regulate gene expression (36). Furthermore, TERRA levels are cell-specific (68) but have not been assessed in cardiomyocytes. As such, I also treated the HPCs in Chapter 5 with a custom TERRA-siRNA but this was defective and hence had to be excluded. Nonetheless, as findings from this Thesis suggest that aberrations in telomere maintenance are sufficient for the development of HPC hypertrophy, the role of other telomeric regulators, such as TERRA telomere repeat-binding factor 1 (TRF1), or tripeptidyl-peptidase 1 (TPP1), in regulating human cardiomyocyte size could be addressed in future knockdown or overexpression studies. As cardiac hypertrophy can occur in response to

hypertension, atherosclerosis, and aortic stenosis (35), elucidating the regulation of cardiomyocyte telomeres could provide insights into the development of heart disease more generally.

Methodological considerations

The use of different methods to measure TL could be necessary in the study of cardiac hypertrophy and the testing of causal hypotheses. For instance, Flow fluorescent in situ hybridization (Flow FISH) uses labelled peptide nucleic acid (PNA) probes specific for telomere repeats and fluorescence generated by flow cytometry (4). The benefits of Flow FISH for the measurement of telomeres in cardiomyocytes is that cell size is also determined allowing for telomere length to be quantified in subsets of cells of based on size. Furthermore, these single-cell measurements provide a population distribution rather than a measure of the average TL for the population, like the T/S ratio method used in this Thesis (11). A population distribution allows for the quantification of short telomeres and minimises the possibility of type 2 errors if changes in TL are present only in a subset of cells (4). Given these benefits, Flow FISH was used in Chapter 4 to determine TL in HPCs with untreated H9c2 cells as the internal controls. Unfortunately, the H9c2 cell line was not a suitable internal control due to the difference in DNA content between rat and human cardiomyocytes. As the recommended internal cell line control was not available due the high cost, this method had to be abandoned.

Telomere-specific PNA probes are also used for quantitative fluorescent in situ hybridization FISH (qFISH) with confocal microscopy (3). Although Flow FISH is highly quantitative and prevents biased sampling, qFISH is considered more sensitive to detect small changes in TL (26, 31) which could be particularly useful in short-term studies where changes in TL are likely to be small. Furthermore, other cell-specific variables, such as surface area (cell size) and proliferative ability, can be detected in conjunction with TL (63),

providing insights into the relationships between telomeres and other cardiac risk factors. As such, Chapter 5 also used qFISH to determine TL in HPCs but the transfer of adherent cells to appropriate slides and obtaining sufficient number of metaphase spreads required for analysis was not possible with the available resources. Indeed, cells with low mitotic indices may not be amenable to the qFISH method (3) and other studies have reported significant reductions in the number of metaphase spreads following BIBR1532 treatment even in rapidly dividing cancer cells (26). Furthermore, inducing mitosis and metaphase arrest can potentially confound experimental treatments as well as bias the results if senescent cells are excluded from analysis (38, 56). Similarly, Flow FISH requires cells to be grown in suspension and in the present Thesis all *in vitro* experiments were performed using adherent cells. In this regard, adherent cells may grow and respond differently to treatments, making it difficult to compare findings from adherent cells. The T/S ratio method, however, does not require additional treatments or specific cell preparations and has been shown to strongly and reliably correlate with TL measurement generated from qFISH (51) and Flow FISH (28).

Conclusions

The Studies undertaken in this Thesis provide new evidence implicating telomeres in the development of cardiac hypertrophy. Decreases in *Tert* expression and telomere length in the heart were found to parallel the development of cardiac hypertrophy in the HHR. TL in circulating leukocytes was also shown to strongly correlate with cardiac TL but only in hypertrophic rat hearts. In addition to the well-established propensity to heart disease, fetal growth restriction and low birthweight reduced cardiac TL in later life in rats. The ability of short-term exercise interventions in adolescence to attenuate cardiac telomere attrition in older rats highlights new ways of approaching cardiac aging and disease prevention.

Of all the methods of inducing hypertrophy in HPCs, only ET-1 shortened HPC telomeres, highlighting a novel role for this agonist in human cardiomyocyte aging. Interestingly, inhibiting telomere maintenance had an even more severe effect on HPC hypertrophy than agonist stimulation. In particular, *TRF2* knockdown induced a molecular and cellular phenotype that closely reflected changes observed in human heart failure (17, 53). In conclusion, the findings from this Thesis suggest an important role for the structure and maintenance of telomeres in regulating cardiomyocyte growth, paving the way for further research in this promising field.

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